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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This program provided an intense training environment with hands-on experience in molecular approaches to marine biology. During the two year program, 57 students participated in concentrated, undistracted courses in the areas of (1) the physiology and molecular biology of algae, (2) the cell biology of early development, and (3) video microscopy and image processing in biology. This intensive training environment resulted in students acquiring new approaches to attack classic problems of marine biology, using technologies of molecular and cellular biology which have previously not been aggessively applied to marine problems. The course environment also redirected many students in their research approaches and additionaly resulted in the publication of three research articles.

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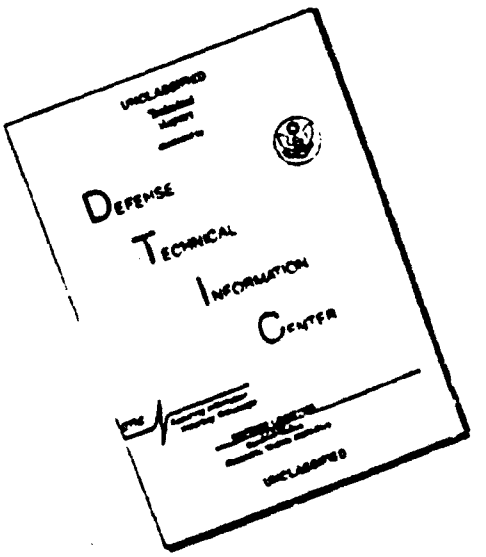

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Final Report on Contract N00014-88-K-0325

Principal Investigators: David Epel and Daniel Mazia

Contractor: Stanford University

Contract Title: Instruction at the Hopkins Marine Station

Dates: 04/01/88 to 03/31/90

A. Student Selection Statistics

A total of 57 students participated in ONR-funded courses at the Hopkins Marine Station during the summers of 1988 and 1989. Applications came from institutions from all over the US, from South America, Japan, Switzerland, Italy, Great Britain and Sweden. The accepted students ranged from advanced undergraduates, students entering graduate programs to students in their early graduate careers, those near completion of their Ph.D.s, post-doctorals and established investigators. The majority of students, however, were graduate students. The acceptance statistics are provided below.

1. Ecophysiology and Molecular Biology of Macrophytes

Year	Applicant Pool	Accepted	ONR Fellows
1988	14	7	3
1989	22	9	9

2. Cell Biology of Early Development

1988	23	14	13
1989	22	14	9

3. Video and Laser Microscopy

1988		(not offered)	
1989	30	13	9

From above, 43 of the 57 students were able to attend with ONR funding; support from Stanford University funds was provided to some of the remaining students who participated and some students were supported by their home institutions.

B. Accomplishments of Previous Training Effort

The appended lecture and laboratory schedules indicate the intensity of the lecture and laboratory aspects of the courses. The "Marine Macrophyte" and "Developmental Biology" courses also involved research projects. At the end of the courses an Annual Meeting (China Point Academy of Sciences Summer Session Symposium) is held at which all of the students present their research findings. The meeting is attended by the students and faculty as well as many guests from Stanford, Monterey Bay Aquarium, UC Santa Cruz, MBARI, Moss Landing Marine Laboratory and other near-by institutions. The titles of these projects are also appended (the appended program for 1989 includes a listing of subtidal ecology research projects which were not part of the ONR program).

The impact of these courses is enormous. The course comments, solicited by the University for each course each summer, were unanimous in praise for the content and impact. In fact some graduate programs (ie. USC, UCLA, etc.) are strongly urging their graduate students in marine sciences to take one of the ONR-Advanced Research Training Programs and particularly those at Hopkins. Other indications of the impact can be seen from the consequences to the

The class of 1989 was truly exceptional. Of the nine students, 8 were in the early stages of their graduate research while one had just completed his doctoral. Alice Goa (USC) is returning to Hopkins this winter to finalize a few aspects of her research on the molecular regulation of nitrogen assimilation algae. She will also be preparing a manuscript for publication on this work. Shelia Oberto (UCLA) examined molecular aspects of cold-shock and is currently exploiting these techniques to study the regulation of symbiont population density in tropical anemones. Sean Fain (UC Santa Barbara) and Haroun Frick (ETH-Zurich) worked together to examine the speciation in Macrocystis, the giant kelp, using RFLP and chloroplast and nuclear DNAs. This work is currently in manuscript form and will be submitted to the Journ. Phycology in the next month. Tenna Michaels (Hawaii) developed the use of plant lectins to identify "recognition molecules" on the surfaces of seagrass leaves that serve as attachment sites for fouling algal spores. She is continuing this line of research for her Ph.D. under the supervision of Dr. Celia Smith, one of the course instructors. Alan Milligan (SUNY-Stony Brook) developed new techniques for the isolation and stabilization of caroteno-chlorophyll proteins from marine algae. He is now using these techniques and others learned in the course to study the formation of "brown tides" in coastal waters for his degree work at Stony Brook. Aljeandro Cabello-Pasini (Stony Brook) and Ian Stupakoff (Stony Brook) are both developing their Ph.D. research around their course research projects. Both will return to Hopkins this summer to follow-up on some of the studies that they initiated in the course.

4. Publications

Poccia, D.W., Pavan, W.J. and Green, G.R. 1990. GDMAP inhibits chromatin condensation but not sperm histone kinase in sea urchin male pronuclei. Exp. Cell Res. 188, 226-234 (1990).

Lauzon, R. and Weissman, I.L. 1990. The paternal centrosome directs the polarity of early pattern formation in the fertilized Ascidia ceratodes egg. In, Invertebrate Reproduction, 5th Internatl. Congr. Invert. Reproduction 5, 131-138 (1990)

Goa, Y., Smith, J.G. and Alberte, R.S. Regulation of nitrate assimilation in Ulva latuca: Evidence for the role of light in induction of nitrate reductase and control by a circadian rhythm. Marine Biology 112, 691-696 (1992)

C. Summer Faculties 1988 and 1989

1. Cell Biology of Early Development

	1988	1989
Resident Faculty:	David Epel, Stanford	David Epel, Stanford
	Daniel Mazia, Stanford	Michael Hadfield, Hawaii
	Dominic Poccia, Amherst	Daniel Mazia, Stanford
		Gerald Schatten, Wisconsin
		Heide Schatten, Wisconsin
		Victor Vacquier, SIO
Visiting Faculty:	Zacheus Cande, Berkeley	Richard Steinhard, Berkeley
	Ellen Dirksen, UCLA	
	Gerald Schatten, Wisconsin	
	James Spudich, Stanford	
	Frank Suprynowicz, Scripps Clinic	
	Robert Swezey, Stanford	

2. Ecophysiology and Molecular Biology of Marine Macrophytes

	1988	1989
Resident Faculty:	Celia Smith, Hawaii	Celia Smith, Hawaii
	Steven Fain, Cal Tech	Stevn Fain, U Chicago
	Richard Zimmerman, U Chicago	Jason Smith, U Chicago
	Jason Smith, U Chicago	Richard Zimmerman, U Chicago
	Robert Smith, U Chicago	Robert Smith, U Chicago
Visiting Faculty:	Elma Gonzales, UCLA	Ralph Quatrano, UNC
	Mimi Koehl, Berkeley	Valerie Vreeland, Berkeley
	Mark Denny, Stanford	Mark Denny, Stanford
	Lenard Muscatine, UCLA	Ladd Johnson, Washington
	Rose Ann Cattolico, Washington	Steve Manley, CSU-Long Beach
	Polly Penhale, NSF	
	Lynda Goff, UC Santa Cruz	

3. Laser and Video Microscopy

1989

Academic Faculty:
Stuart Thompson (Stanford)
Ron Vale (UCSF Medical School)
James Spudich (Stanford Medical School)
Sam Wong (Stanford)
Mike Cahalan (UC Irvine Medical School)
Steve Finkbeiner (Yale School of Medicine)
Richard Tsein (Stanford Medical School)
Lubert Stryer (Stanford Medical School)
Stephen Smith (Stanford Medical School)
Anna Spudich (Stanford Medical School)
Dan Madison (Stanford Medical School)

Commerical Faculty: V. Argiro (Vital Images)
K. Boydston (Megavision Inc.)
T. Bruchman (Photometrics Ltd.)
M. Delay, Ph.D. (Axon Instr. Inc.)
K. Hendricks (Nikon Instr.)
R. Haugland, Ph.D. (Molecular Probes Inc.)
T. Inoue (Universal Imaging)
T. Knightly
P. Moore (Molecular Probes Inc.)
M. Shelly (Sea Studios)
E. Snyder (Olympus Microscopes)
P. Steinbach (ETM Systems)
A. Trimble (Silicon Graphics)
R. Wick (Photonic Microscopy Inc.)
P. Wong (Newport Corp.)

D. ONR Molecular Marine Biology Fellows

1. Ecophysiology and Molecular Biology of Marine Macrophytes

1988

Josef D. Ackerman (Cornell University)
Linda A. Franklin (Duke University)
Stuart Slaven (Univ. of Arkansas School for Medical Sciences)

1989

Alejandro Cabello-Pasini (SUNY at Stony Brook)
Sean B. Fain (UC Santa Barbara)
Haroun Frick (ETH-Zurich)
Yu (Alice) Gao (USC)
Teena Michael (Univ. of Hawaii)
Allen J. Milligan (SUNY, Stony Brook)
Sheila R. Oberto (UCLA)
Ian Stupakoff (SUNY, Stony Brook)
Masami Watanabe (U Tokyo)

2. Cell Biology of Early Development

1988

Mark W. Haffer (UC Davis)
Navdeep S. Jaikaria (NY Medical College)
Minas Kocamoglu (California State University-Fullerton)
Robert Lauzon (Stanford University Medical School)
Sandy K.S. Luk (University of Manitoba)
David Nagajski (University of Sussex)
William J. Pavan (Johns Hopkins University School of Medicine)
Clara A. Pinto Correia (Lisbon Medical School)
Ellen M. Popodi (Marquette University)
Gustavo R. Rosania (Stanford University)
Kristin F. Thomas (California State University-Fullerton)
Marie A. Vodicka (Amherst College)
Harry Witchel (UC Berkeley)

1989

Marguerite Chow (U Pennsylvania)

1989 Pilar Ducci (Pontifical Catholic Univ., Chile)
Carolyn M. Fleming (Vanderbilt Univ.)
Jan Hoh (Caltech)
Deborah A. Jacobs (Albert Einstein College of Medicine)
Suresh J. Jesuthasan (Stanford)
Andrea Lanctot (Stanford)
Terese Rakow (Iowa State Univ.)
Siming Wang (Brown Univ.)

3. Video and Microscopy

1989

Susan DeMaggio (UC Irvine)
Lise S. Eliot (New York State Psychiatric Institute)
Deanna Frost (Univ. of Oregon)
Robert Grad (Univ. of Hawaii)
Mary Hagedorn (Univ. of Oregon)
Fady Malik (UCSF)
Stephen A. Stricker (UC Santa Cruz)
Ben Stowbridge (Yale Univ.)
Susan Treves-Zorzato (Univ. of Padova, Italy)

Appendix

Lecture/Laboratory Schedules

CELL BIOLOGY OF EARLY DEVELOPMENT: THE CELL CYCLE

June 13-July 15, 1988
Hopkins Marine Station
David Epel, Daniel Mazia and Dominic Poccia, Instructors

Lectures will be in Agassiz 11, typically from 9:00 am to noon. Labs will begin ~1:00--1:30 pm (depending on when lecture is over). On days of field trips, the lecture will be later (time to be announced).

Week 1	June 13	Cell activation	D. Epel
	14	Cell activation	D. Epel
FIELD TRIP	15	Cell activation	D. Epel
		Cell permeabilization	Robert Swezey (HMS)
FIELD TRIP	16	Cell cycle	D. Mazia
	17	Cell cycle	D. Mazia
Week 2	June 20	Mitotic apparatus	D. Mazia
	21	Cytoskeleton/Cytokinesis	James Spudich(Stanford)
	22	Chromosome movement	Zacheus Cande (UCB)
	23	Mitotic chromosome condensation	D. Poccia/D. Mazia
	24	----------	
Week 3	June 27	Spermatogenesis/Pronuclear activation	D. Poccia
	28	Histones in the Cell Cycle	D. Poccia
	29	Ciliogenesis	Ellen Dirksen (UCLA)
	30	Cell Organization	Gerald Schatten(Wisc)
FIELD TRIP-July 1		Protein Phosphorylation in the Cell Cycle	Frank Suprynowicz (Scripps Clinic).
Week 4	July 4	----------	
	5	RESEARCH PROJECTS	
	6	" "	
	7	" "	
	8	" "	
Week 5	July 11	" "	
	12	" "	
	13	" "	
	14	" "	
	15	CLASS SYMPOSIUM	

CELL BIOLOGY OF EARLY DEVELOPMENT: THE CELL CYCLE

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	24	.....	
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	5	RESEARCH PROJECTS	
	6	" "	
	7	" "	
	8	" "	
Week 5	July 11	" "	
	12	" "	
	13	" "	
	14	" "	
	15	CLASS SYMPOSIUM	

Summer 1968 | ECOPHYSIOLOGY & CELL BIOLOGY OF MARINE MACROPHYTES | 142H

Date	Lecture Schedule	Lecturer
	WEEK I	
Mon. June 13	The Inter- and Subtidal Zones	C. Smith
	The Chlorophyta	C. Smith
Tues. June 14	The Rhodophyta	C. Smith
	The Rhodophyta	C. Smith
Wed. June 15	The Phaeophyta	C. Smith
	The Kelps	C. Smith
Thurs. June 16	Intertidal Transect	
	The Seagrasses	R. Alberte
2:30 p.m.	Monterey Bay Aquarium Tour	
Fri. June 17	More Intertidal Field Work	
11:00 a.m.	Optical Properties of the Water Column	R. Zimmerman
	Light Phenomena: Pigments and Photoreception	R. Alberte
	HOPKINS LECTURE	
4:00 p.m.	Women in Science	P. Penhale
Sat. June 18	Big Sur Field Trip (8:00 to ca. 2:00)	

WEEK II

Mon. June 20	Molecular Tools for Studying Adaptation	R. Alberte
	Marine Symbioses	L. Muscatine
Tues. June 21	Targeting and Cell Wall Synthesis	E. Gonzalez
	Pigment-Proteins & the Photosynthetic Unit	R. Alberte
Wed. June 22	Cell & Molecular Biology of Chloroplasts	R. Alberte
	Light Reactions in Photosynthesis	R. Alberte
Thurs. June 23	Applications of DNA Technologies to Algae	S. Fain
	Light Adaptation in Algae	R. Alberte
Fri. June 24	DNA Polymorphisms: Markers for Speciation	S. Fain
	Photosynthetic Carbon Metabolism	R. Alberte
evening	Research Project Discussions	
Sat. June 25	Ano Nuevo Field Trip (10:00 to ca. 3:00)	

WEEK III

Mon. June 27	Carbon Metabolism & Partitioning	R. Alberte
	Nutrient Dynamics in Algae	R. Zimmerman
Tues. June 28	Nitrogen Assimilation and Metabolism	R. Zimmerman
	Integration of Metabolism and Cell Processes	R. Alberte
Wed. June 29	Immunological Methods for Macrophytes	R. Alberte
	HOPKINS LECTURE	
4:00 p.m.	Molecular Approaches to Algal Phylogenies	R. Cattolico
Thurs. June 30	Stress in the Intertidal	C. Smith
	Salinity and Temperature Stress	C. Smith
Fri. July 1	Fluid Dynamics of the Inter- & Subtidal	M. Denny
	Parasitism in Red Algae	L. Goff
Sat. July 2-	Elkhorn Slough Field Trip (10:00 to ca. 3:00)	

WEEK IV

RESEARCH PROJECTS

Mon. July 4	Picnic	
Tues. July 5 4:00 p.m. Fisher Hall	VAN NIEL MEMORIAL LECTURE Silicon and Life: What the Diatom Can Tell Us	B. Volcani
Weds. July 6 (9:00 a.m.)	Environmental Control of The Cell Cycle	J. Smith
Thurs. July 7	Life in reducing Sediments	R. Smith

WEEK V

RESEARCH PROJECTS

Mon. July 11	Flow, Flapping and Photosynthesis: The Role of Undulate Blades	M. Koehl
July 14-15	Research Project Reports - MMS Annual Meeting	

LABORATORY SCHEDULE

WEEK I

Mon. Jun. 13	Laboratory - Green Algae
Tues. Jun 14	Laboratory - Red Algae
Weds. Jun 15	Laboratory - Brown Algae
Thurs. Jun 16	Field - Intertidal Transect, Data Analysis (Lotus)
Fri. Jun 17	Field - Intertidal Work, Data Discussions
	Pigment Analyses and Spectrophotometry
Sat. Jun 18	Field Trip to Big Sur

WEEK II

Mon. Jun 20	Oxygen Exchange Technologies/Spectrophotometry
Tues. Jun 21	Measurement of Reaction Centers, PSU sizes
Weds. Jun 22	Protoplast Isolation
Thurs. Jun 23	Isolation and Purification of DNA and RNA
Fri. Jun 24	DNA Restriction Mapping
Sat. Jun 25	Field Trip - Ano Nuevo

WEEK III

Mon. Jun 27	Nitrate Assimilation - Nitrate Reductase
Tues. Jun 28	Ammonium Assimilation - Glutamine Synthetase
Weds. Jun 29	Protein Isolation and Separations/Western Blotting
Thurs. Jun 30	In situ Immuno-localizations
Fri. Jul 1	Fluorescence Microscopy
Sat. Jul 2	Field Trip - Elkhorn Slough

WEEKS IV & V

Jul 5-13	RESEARCH PROJECTS
Jul 14-15	Research Project Presentations

TENTATIVE LECTURE/FIELD TRIP SCHEDULE

BIOLOGY 136--CELL BIOLOGY OF EARLY DEVELOPMENT
Summer 1989

June 19	9:00 AM - Lecture - Introduction to course (D. Mazia) Reproductive patterns (M. Hadfield) 1:00 PM - Lecture - Echinoid development (D. Epel)
June 20	6:00 AM - Field trip 9:30 AM - Lecture - Patterns of early development (Hadfield)
June 21	6:00 AM - Field trip 9:30 AM - Lecture - Gastropod development (Hadfield)
June 22	7:00 AM - Field trip 10:00 AM - Lecture - Polychaete development (Hadfield)
June 24	9:00 AM - Development in selected groups (Hadfield)
June 26	Lecture - 9:00 AM - Fertilization and the initiation of embryonic development, part 1 (Epel)
June 27	9:00 AM - Fertilization/egg activation, part 2 (Epel)
June 28	9:00 AM - Sperm activation/motility (Vacquier)
June 29	9:00 AM - Sperm activation/acrosome reaction (Vacquier)
June 30	9:00 AM - Sperm-egg attachment/other aspects of egg activation (Vacquier/Epel)

TENTATIVE LECTURE/FIELD TRIP SCHEDULE (Cont.)

BIOLOGY 136--CELL BIOLOGY OF EARLY DEVELOPMENT

July 3	9:00 AM - Mitotic cycle (Mazia)
July 4	Holiday
July 5	9:00 AM - Fertilization/pronuclear fusion (Schatten)
July 6	9:00 AM - New methods: microscopy and image processing (Schatten)
July 7	9:00 AM - Centrosomes (Mazia, Schatten and Schatten) 4:00 PM - Station seminar - "Gene Regulation in sea urchin development" (Eric Davidson, Cal Tech)
July 10 - July 20	RESEARCH PROJECTS
July 11	4:00 PM - Van Niel Memorial Lecture - by Harlyn Halvorson, MBL, "Polyphosphate: A Storage Reserve of Phosphate or Energy?"
July 17	9:00 AM - "Calcium during the Cell Cycle" (Richard Steinhardt, UC Berkeley)
July 21	Reports on Research Projects

Summer 1989

ECOPHYSIOLOGY & CELL BIOLOGY OF MARINE MACROPHYTES

142H

Date	Lecture Schedule	Lecturer
	WEEK I	
Mon. June 19	The Inter- and Subtidal Zones	C. Smith
	The Chlorophyta	C. Smith
Tues. June 20	The Phaeophyta	C. Smith
	The Phaeophyta	C. Smith
Wed. June 21	The Rhodophyta	C. Smith
	The Rhodophyta	C. Smith
Thurs. June 22	Seagrasses	R. Alberte
4:00	Hopkins Summer Seminar, Fisher Lecture Hall	D. Schiel
Fri. June 23	Intertidal Field Work Discussion	Student Teams
	Algal pigments	R. Alberte
	Light Phenomena: Light harvesting	R. Alberte
Sat. June 24	Big Sur Field Trip (depart Hopkins @ 8:30 return ca. 3:00)	

WEEK IV

RESEARCH PROJECTS

Thurs. July 12	Immunological studies of algal cell walls (lecture and lab demo)	V. Vreeland

WEEK V

RESEARCH PROJECTS

July 21	Research Project Reports - HMS Annual Meeting

Thurs. July 6	DNA probe labelling techniques; Filter hybridization RNA fractionation
Fri. July 7	Fluorescence Microscopy
Sat. July 8	Field Trip - Elkhorn Slough

WEEKS IV & V

Jul 10-20	RESEARCH PROJECTS
Jul 21	Research Project Presentations

LASER & VIDEO MICROSCOPY

Hopkins Marine Station and
Dept. of Molecular & Cellular Physiology
Stanford University
Summer 1989

(Preliminary Schedule)

Mon. July 24TH

9-11	Welcome to HMS & mutual introductions - Thompson
11-12	Introduction to electronic light microscopy (ELM) - Smith
1	Begin lab work
4-5:30	SJS lab- seminar sampler

Tues.

9-10	Fundamentals of ELM (Electronic Light Microscopy) - SJS
10	Video fundamentals - Wick
4	Fluorescent probes I - Haugland

Wed.

9-10	Fundamentals of ELM - SJS
10	Fluorescent probes II - Haugland
4	Glutamate and Ca in astrocytes - Finkbeiner

Thur.

9-10	Fundamentals of ELM - SJS
10	3-dimensional image processing - Argiro
11	Ca in neurons - Tsein
4	Physiology of lymphocytes - Mike Cahalan

Fri.

9-10	Fundamentals of ELM - SJS
10	Laser technology - J.R. Young
4	CCD cameras - Bruchman

Sat.

Lab day

Mon. July 31.

9-10	Fundamentals of ELM - SJS
10	Introduction to image processing software, user interfaces - T. Inoue
4	Ca & Neuronal growth - Kater

Tues.

9-10	Fundamentals of ELM - SJS
10	The video processing pipeline - Steinbach
4	Microtubule based motility - Vale

Wed.

9-10	Frontiers of ELM - SJS
10	Fluorescent probe frontiers - Haugland
4	Brain slices - Madison

Thur.

9-10	Video editing - Mark Shelly
10	Optical recording of membrane potential - Lev Ram
4	Mechanisms of Ca oscillations - Stryer

Fri.

9-10	Frontiers of ELM - SJS
10	Microspectrophotometry - Delay
4	Cortical actin states - A. Spudich

Sat.

Lab day

Appendix

Programs, Annual Research Symposia

CHINA POINT ACADEMY OF SCIENCES

HOPKINS MARINE STATION

FOURTH ANNUAL

SUMMER SESSION RESEARCH SYMPOSIUM

FRIDAY, JULY 15, 1988

FISHER HALL

CHINA POINT ACADEMY OF SCIENCES
HOPKINS MARINE STATION

FIFTH ANNUAL
SUMMER SESSION RESEARCH SYMPOSIUM

FRIDAY, JULY 21, 1989

FISHER HALL

11:10-11:20	Caroline Clevenger	Serpula vermicularis: Distribution and microhabitat within a kelp forest.
11:20-11:30	Bill Hoese	Cytochalasin-induced motility in sea urchin eggs.
11:30-11:40	Deborah Jacobs	Is ATP required for yellow crescent formation in Ascidea cestoda?
11:40-11:50	Alejandro Cabello-Pasini	Comparative ammonium assimilation with light and dark carbon fixation for Macrocystis pyrifera.
11:50-12:00	Allen Milligan	Isolation and characterization of light-harvesting pigment protein complexes from Macrocystis pyrifera.
12:00-1:00		LUNCH BREAK
SESSION 3		Chairperson: Richard Zimmerman
1:00-1:10	Carolyn Fleming	Chromosome cyles in sea urchin eggs following the inhibition of protein synthesis by emetine: polytene chromosomes?
1:10-1:20	Xudong Yin	Effect of E-64, an inhibitor of protease activity, on the cell cycle of sea urchin eggs
1:20-1:30	Jim Owens	Distribution of Calliostoma ligatum with regard to certain abiotic and biotic factors.
1:30-1:40	Diana Olson	Habitat associations and the decorating behavior of Loxorhyncus crispatus.
1:40-1:50	Teena Michael	Characterization of lectin binding proteins from cell walls of Zostera marina.
1:50-2:00	Ian Stupakoff	Responses by roots of Zostera marina to hydrogen sulfide and implication for respiration.
2:00-2:10	Alice Gao	Light and dark regulation of nitrate reductase in Ulva.
2:10-2:20	Siming Wang	The cell cycle is activated by the calcium ionophore A23187.
2:20-2:30	Jean Liu	The monopolar mitotic cycle in sea urchin eggs.
2:30-2:50		COFFEE/TEA BREAK

SESSION 4 Chairperson: David Epel

2:50-3:00	John Hodge	Distribution and abundance of *Membranipora membranacea* on *Macrocystis pyrifera*.
3:00-3:10	Catherine O'Riordan	Colony size and oriented growth of *Membranipora membranacea* on *Macrocystis pyrifera*.
3:10-3:20	Sean Fain	Restriction fragment polymorphisms in populations of *Macrocystis pyrifera* from exposed and sheltered locations.
3:20-3:30	Haroun Frick	Interspecies variation in the genus *Macrocystis*: restriction analysis of nuclear DNA.
3:30-3:40	Teresa Rakow	BCECF as a pH indicator in eggs.
3:40-3:50	Suresh Jesuthasan	Centrosome movement after nuclear fusion.
3:50-4:00	Jeff Light & Jennifer Chaffee	Size and distributional dynamics of *Pugettia producta*.
4:00-4:10	Becky Hughes	The effects of water movement on the distribution and orientation of *Doriopsilla albopunctata*.
4:10-4:20	Monique Tenarson	Distribution of the sexes and asexes of *Gigartina corymbifera* and *Rhodymenia* spp.

4:20-4:30 Concluding Remarks: Randall Alberte

EXPERIMENTAL CELL RESEARCH 188, 226–234 (1990)

6DMAP Inhibits Chromatin Decondensation but Not Sperm Histone Kinase in Sea Urchin Male Pronuclei

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Treatment of sea urchin eggs for 10 min prior to fertilization with the kinase inhibitor 6DMAP (6-dimethylaminopurine) reversibly inhibits swelling and loss of conical morphology of the male pronucleus. Male pronuclei inhibited with 1 m*M* 6DMAP for 25 min undergo phosphorylation of Sp H1 and Sp H2B histones as fully as do control nuclei. Therefore, Sp histone kinase, whose target sequences resemble those of the M-phase histone kinase, is not inhibited by 6DMAP, and Sp histone phosphorylation, although it may be necessary, is not sufficient for chromatin decondensation. © 1990 Academic Press, Inc.

INTRODUCTION

As a consequence of events occurring late in spermatogenesis, the spermatozoon nucleus becomes genetically quiescent, usually acquiring one or more distinctive DNA-binding proteins and highly condensed chromatin. Following fertilization, chromatin decondensation and changes in nucleoprotein composition transform the inert sperm nucleus into a male pronucleus capable once again of participating in replication, transcription, and mitosis [1].

Male pronuclear development in the sea urchin involves several changes in nucleoprotein composition. The earliest of these occur almost immediately upon fertilization and result in modification of the two male germ-line-specific histone classes (Sp H1 and Sp H2B) and the acquisition of histone CS H1 from the egg. Later changes include the loss of modified Sp H1 and the accumulation of histones of CS H2A, CS H2B, and H3* from the maternal storage pool. The later events result in the male chromatin histone composition duplicating that of the female. However, the early modifications of Sp histones may be sufficient for functional reactivation, since chromatin of similar composition is present during spermatogenesis when mitosis, meiosis, replication, and transcription all occur [2].

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The two germ-line-specific Sp histone classes modulated in their DNA-binding capacity by phosph ylation [3, 4]. The multiple sites of phosphorylation clude rare tetrapeptide sequences of the composit serine–proline adjacent to two basic amino acids (lys and/or arginine). Identical sites are found in the C-t minal regions of H1 histones where they are targets the M-phase-specific histone kinase [5]. Several s sites occur in other nucleic acid-binding proteins wh package or regulate condensed chromatin such as he titis core antigens, chicken sperm basic protein, mur caronavirus nucleocapsid protein, wheat germ hist H2A [5], mussel sperm nuclear protein ϕ3 [6], and man chromosome condensation regulator RCC1 [7. In sea urchin Sp histones, the sequences confer dicted β-turn structure upon the *N*-terminal reg which may facilitate their binding to linker D [3, 5, 9].

The unphosphorylated forms of Sp histones are c acteristic of mature sperm and may exist briefly onl late spermatids and early male pronuclei. We previo postulated a role for their unphosphorylated *N*-term arms in chromatin condensation or stabilization of ture sperm chromatin [3]. However, a role in bulk c matin condensation was made unlikely by the obse tion that virtually all the compaction of male germ chromatin during spermiogenesis takes place befor dephosphorylation of the Sp histones [2]. After ferti tion, phosphorylation appears to slightly precede de densation of male pronuclear chromatin, although rapidity of both responses makes their relative ti somewhat imprecise [3].

To delineate more closely the relationship betwee histone phosphorylation and male pronuclear chr tin decondensation, we sought an inhibitor of pronu development. Few treatments seem to reversibly pre decondensation. Of a wide range of metabolic and c inhibitors tested, only cold and *N*-ethylmaleimide tically slowed the rate of pronuclear swelling [10]. former was effective at 20°C below normal and wa versible after 20 min; the latter was not reversible the same period.

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In the hope that prevention of Sp histone phosphorylation would result in blockage of decondensation we considered kinase inhibitors. Recent work on the purine analog 6-dimethylaminopurine (6DMAP) suggests that this drug might exert its effects through kinase inhibition [11]. 6DMAP is a potent cleavage inhibitor in sea urchin and surf clam eggs while leaving protein synthesis, cyclic AMP phosphodiesterase activity, and cAMP levels unaffected [12, 13]. Added just after fertilization, 6DMAP blocks sea urchin eggs at prophase of the first mitosis [14]. Under these conditions, it does not alter protease, phosphatase, or synthesis of cyclin [11]. It may block activation of maturation promoting factor (MPF), but not the action of activated MPF [14].

Since MPF has recently been found to contain a M-phase-specific H1 kinase [15, 16] and the sites of multiple phosphorylation of sea urchin Sp histones include those phosphorylated by the M-phase histone kinase [5, 7], 6DMAP was expected to block conversion of Sp histones to their modified forms following fertilization. Instead we find that 6DMAP at concentrations sufficient for full inhibition of cell cycle events does not block phosphorylation of Sp histones in male pronuclei. In spite of the apparently normal histone modification, however, pronuclear chromatin decondensation is fully inhibited, suggesting that male pronuclear development is a multistep process in which Sp histone phosphorylation, although it may be necessary, is not sufficient for chromatin decondensation.

MATERIALS AND METHODS

Strongylocentrotus purpuratus were purchased from Marinus, Inc. Long Beach, CA). Eggs were inhibited by suspension in various concentrations of N_6,N_6-dimethyladenine [6-dimethylaminopurine] Sigma, St. Louis, MO) in artificial seawater for 10 min prior to fertilization. For monospermic fertilization, <1/100 volume of dilute sperm suspension was added to a 1% (v/v) suspension of eggs. Fertilized eggs remained in the presence of 6DMAP until fixation. For reversibility experiments, eggs were collected by hand centrifugation and washed three times with 50 vol of seawater. For polyspermy experiments, unfertilized eggs were treated with 6DMAP in N8 (30 m*M* NH_4Cl in seawater, pH 8.3) for 10 min with settling, and the supernatant was drawn off and replaced with a concentrated sperm suspension in 6DMAP-seawater containing 3 m*M* aminotriazole. Fertilized eggs were allowed to settle and washed with 6DMAP-seawater to remove excess sperm. All cultures and washes were performed at 15°C. Inorganic phosphate (^{32}P orthophosphate, carrier-free in acid free aqueous solution, 8 mCi/ml, Amersham) was added to cultures (0.1 mCi/ml) from 20–30 min postfertilization.

Male pronuclei were isolated as previously described [18]. Eggs were screened through 53 μm Nitex and washed twice to remove fertilization membranes and sperm. Histone extraction and gel electrophoresis were performed as previously described [18].

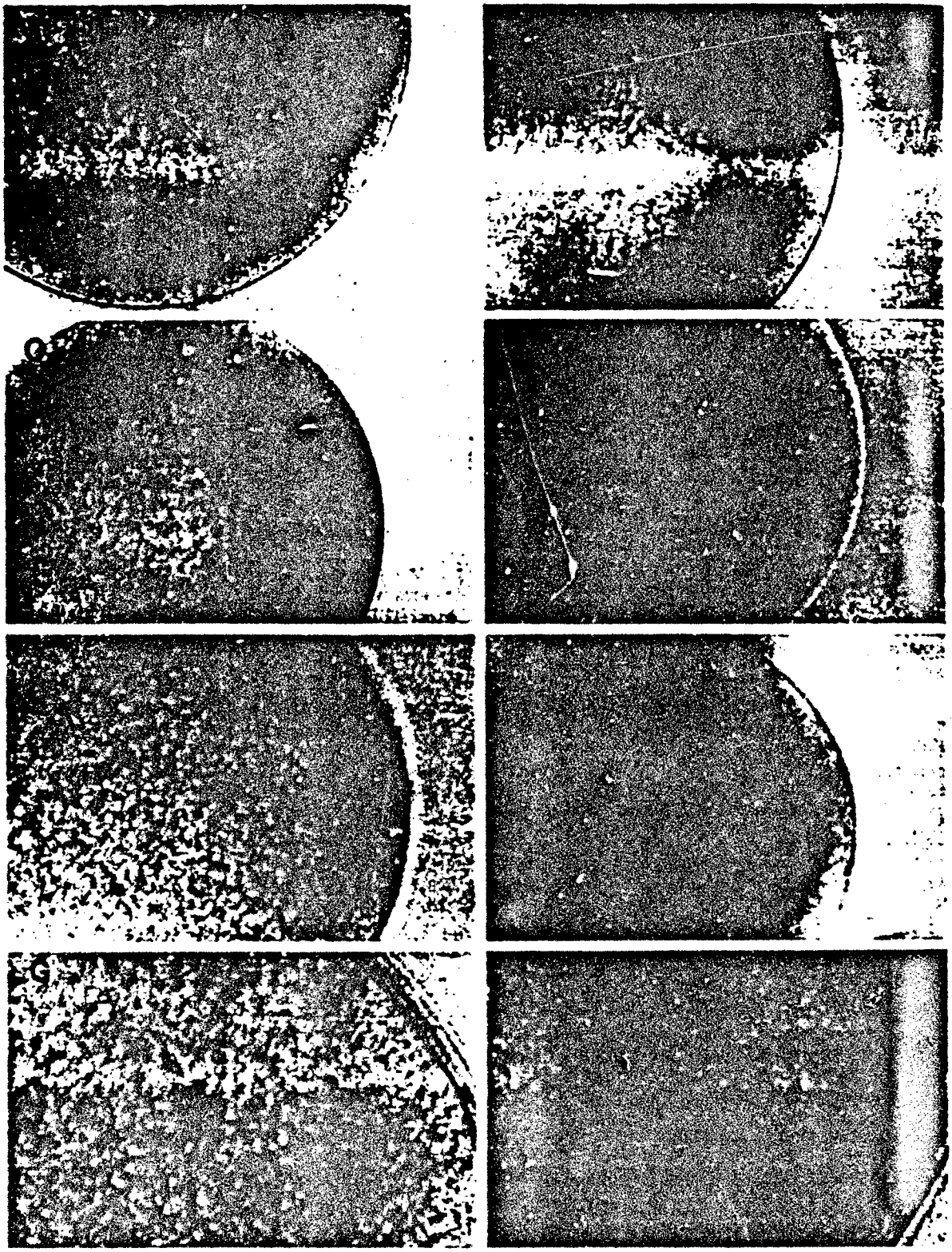
Eggs were fixed in 3:1 ethanol:acetic acid. Aliquots were allowed to dry on slides. Eggs were stained with 2% aceto-orcein-75% acetic acid. Average degree of polyspermy ($\bar{n}$) was calculated by averaging the number of male pronuclei per egg for 25 eggs taken from several different regions of the slide.

RESULTS

Effect of 6DMAP on male pronuclear decondensation in monospermic eggs. Male pronuclear decondensation to a uniformly euchromatic, spherical form is complete by 10–12 min in *S. purpuratus* at 15°C [19]. Loss of conical morphology takes only 1–2 min. Eggs were treated with varying concentrations of 6DMAP to find the minimal concentration which would fully inhibit male pronuclear decondensation. Unfertilized eggs were suspended for 10 min in 6DMAP-seawater, then fertilized. Apparently normal fertilization envelopes appeared in >95% of the eggs at all concentrations of 6DMAP tested. Fertilized eggs were left in inhibitor until fixation at 30 min postfertilization. By this time, all male pronuclei in control eggs had decondensed (Fig. 1A). At the lowest concentrations of 6DMAP used (12, 120 μ*M*), male pronuclei became spherical but did not swell as fully as those of controls (Figs. 1B and 1C). At 300 μ*M* 6DMAP nuclei became spherical but remained condensed and darkly staining (Fig. 1D). At 600 μ*M* 6DMAP, male pronuclei were either spherical or conical but condensed (Fig. 1E). At 2400 μ*M* 6DMAP, male pronuclei remained conical in shape and appeared localized in the egg cortex (Fig. 1F).

Sperm remained viable for at least 25 min in 6DMAP. To test if 6DMAP-treated fertilized eggs were viable, the inhibitor was removed at 20 min postfertilization. Nuclear swelling was evaluated at 40 min. Nuclei previously inhibited at 600 μ*M* (Fig. 1G) decondensed in the absence of inhibitor (Fig. 1H), whereas unwashed controls remained as condensed as at 20 min (not shown). Moreover, eggs recovered and developed after wash out. In one experiment, 94% of 600 μ*M* 6DMAP-inhibited eggs went on to cleave. In a second experiment, 75% of eggs that had been inhibited with 600 μ*M* 6DMAP progressed to blastula and 50% to gastrula. Since 600 μ*M* 6DMAP was effective for preventing decondensation but variable in maintaining conical morphology, 1 m*M* 6DMAP was used for most subsequent experiments. At 1 m*M*, 80% of inhibited eggs cleaved after washout at 20 min, but few reached blastula.

Effect of 6DMAP on male pronuclear decondensation in polyspermic eggs. Since polyspermic eggs are required to facilitate electrophoretic analysis of histone transitions in male pronuclei [20], the effects of 6DMAP were also evaluated in polyspermic eggs. Unfertilized eggs were treated with 1 m*M* 6DMAP-N8 for 10 min, then polyspermically fertilized and cultured in 1 m*M* 6DMAP-seawater. Routinely all male pronuclear decondensation was inhibited, and nuclei remained conical even at degrees of polyspermy as high as 25 male pronuclei per egg. Washout of the drug resulted in the transition from conical to spherical nuclei by 40 min as in monospermic eggs (not shown).



Since the male nuclei appeared to be close to the surface of the egg, it was possible that nuclei scored as conical and condensed were not actually in the egg cytoplasm and that the supernumerary sperm only fused with the egg upon removal of the drug. To distinguish fertilizing sperm from those attached to the surface, we used the technique of Hinkley *et al.* [21]. Unfertilized eggs were prelabeled for 10 min with 1 μg/ml of the DNA-binding fluorescent dye Hoechst 33334, washed twice with seawater, treated with 1 m*M* 6DMAP-N8, and fertilized as above. The egg cytoplasm serves as a reservoir of Hoechst which is taken up by fusing sperm. Figure 2B shows that multiple conical male pronuclei are fluorescent and therefore incorporated into egg cytoplasm, but that the large numbers of unincorporated sperm nuclei surrounding the egg (Fig. 2A) are not fluorescent. The fluorescent nuclei eventually became spherical but were still condensed by 4 h postfertilization in the continued presence of the inhibitor (not shown). Thus 6DMAP appears to greatly delay rather than fully block both the loss of conical morphology and swelling of male pronuclear chromatin.

Fertilization normally sets in motion a linked program of events distinguished as early and late [22]. At least some early events are triggered in the presence of 6DMAP since fertilization envelope formation, normally caused by transient Ca^{2+} release in the cytoplasm, was observed. Ammonia treatments at different pH values can initiate various late events including M-phase histone kinase cycling, bypassing the early events [22–24]. If 6DMAP blocks an early event which in turn triggers a later event required for pronuclear decondensation, activation with ammonia prior to exposure to 6DMAP and fertilization might bypass the 6DMAP block. To test this, unfertilized eggs were treated with N8 or N9 (seawater adjusted to pH 9.3 with NH_4OH) for 10 min, then for 10 min in N8 or N9 containing 1 m*M* 6DMAP and subsequently polyspermically fertilized. In both cases male pronuclei remained conical up to 60 min postfertilization as did the control nuclei from eggs receiving only seawater instead of NH_4OH (N9 results shown in Figs. 2C and 2D). Therefore neither treatment was effective in reversing the 6DMAP blockade.

Effect of 6DMAP on male pronuclear histone phosphorylation. To determine if 6DMAP also blocked the male pronuclear histone transitions believed to be required for chromatin decondensation, pronuclei were isolated from polyspermic eggs blocked with 6DMAP. Eggs were pretreated with N8-1 m*M* 6DMAP for 10 min and fertilized with sperm in 1 m*M* 6DMAP-seawater. At 10 min postfertilization the fertilized egg suspension was screened through 53-μm Nitex to remove the fertilization envelopes and attached sperm, and washed twice with large volumes of 1 m*M* 6DMAP-seawater. At 25 min, eggs were spun through a layer of 1 *M* glucose-6DMAP at 4°C to remove traces of unincorporated sperm and seawater. A small aliquot was fixed (35 min postfertilization), the rest of the eggs were lysed, and the pronuclei were isolated. Fixed samples (Figs. 2E and 2F) showed male pronuclei from inhibited eggs were still conical and unswollen after the glucose wash (at the time of egg lysis) whereas those from uninhibited eggs remained spherical and decondensed.

Figure 3 shows the electrophoretic results of two experiments. All cultures were fertilized to the same degree (13–14 male pronuclei/egg). In Fig. 3B, male pronuclear histones from a culture inhibited with 600 μ*M* 6DMAP is shown compared to the untreated control (Fig. 3A). In this sample, male pronuclei showed some degree of rounding up (see Fig. 1E). In Figs. 3C and 3D, preparations from parallel samples inhibited in 1 and 3 m*M* 6DMAP are shown. In these samples, male pronuclei remained conical and unswollen (see Fig. 1F).

All electrophoretic patterns are very similar. The Sp histones are virtually entirely in the phosphorylated forms N (from Sp H1) and O/P (from the similar variants Sp H2B-1 and Sp H2B-2) at all but the highest 6DMAP concentration. Arrows indicate the electrophoretic positions of unmodified Sp histones. The ratio of O/P to the other core histones is close to 1 for inhibited or control samples, except for the 3 m*M* sample (Fig. 3D). CS H1 is efficiently incorporated into nuclei at all 6DMAP concentrations. Therefore essentially complete conversion of Sp histones to their modified forms takes place in the presence of 6DMAP in male pronuclei whether or not they have undergone shape change or swelling.

Some minor differences are detectable. There is a higher ratio of the Sp H1-derived protein N to core histones at the two highest 6DMAP concentrations, indicating removal of N from pronuclear chromatin may be slightly less efficient under these conditions. Traces of the unphosphorylated Sp H1 (probably <5%) are also present in these two samples. The most striking difference occurs at 3 m*M* 6DMAP in which conversion of Sp H2B is less extensive than conversion of Sp H1, perhaps proceeding to only 50% as judged by the ratio of O/P to other core histones. A heavy spot is present where Sp

FIG. 1. Effects of 6DMAP on male pronuclear decondensation in monospermic sea urchin eggs. (A-F) Dosage effects. (A) 0 μ*M*, (B) 20 μ*M*, (C) 120 μ*M*, (D) 300 μ*M*, (E) 600 μ*M*, (F) 2400 μ*M* 6DMAP. Eggs were treated for 10 min prior to fertilization, fertilized and cultured in the presence of inhibitor for 30 min, then fixed and stained with aceto-orcein. Arrow indicates the weakly staining male pronucleus from the uninhibited control. All pictures at the same magnification. (G, H) Reversibility. A culture treated as above with 600 μ*M* 6DMAP was divided in two at 20 min postfertilization, and the drug was washed out of one culture. (G) Fixation at 20 min; (H) fixation at 40 min of culture washed at 20 min. Pictures taken at approximately 3× greater magnification than (A-F).

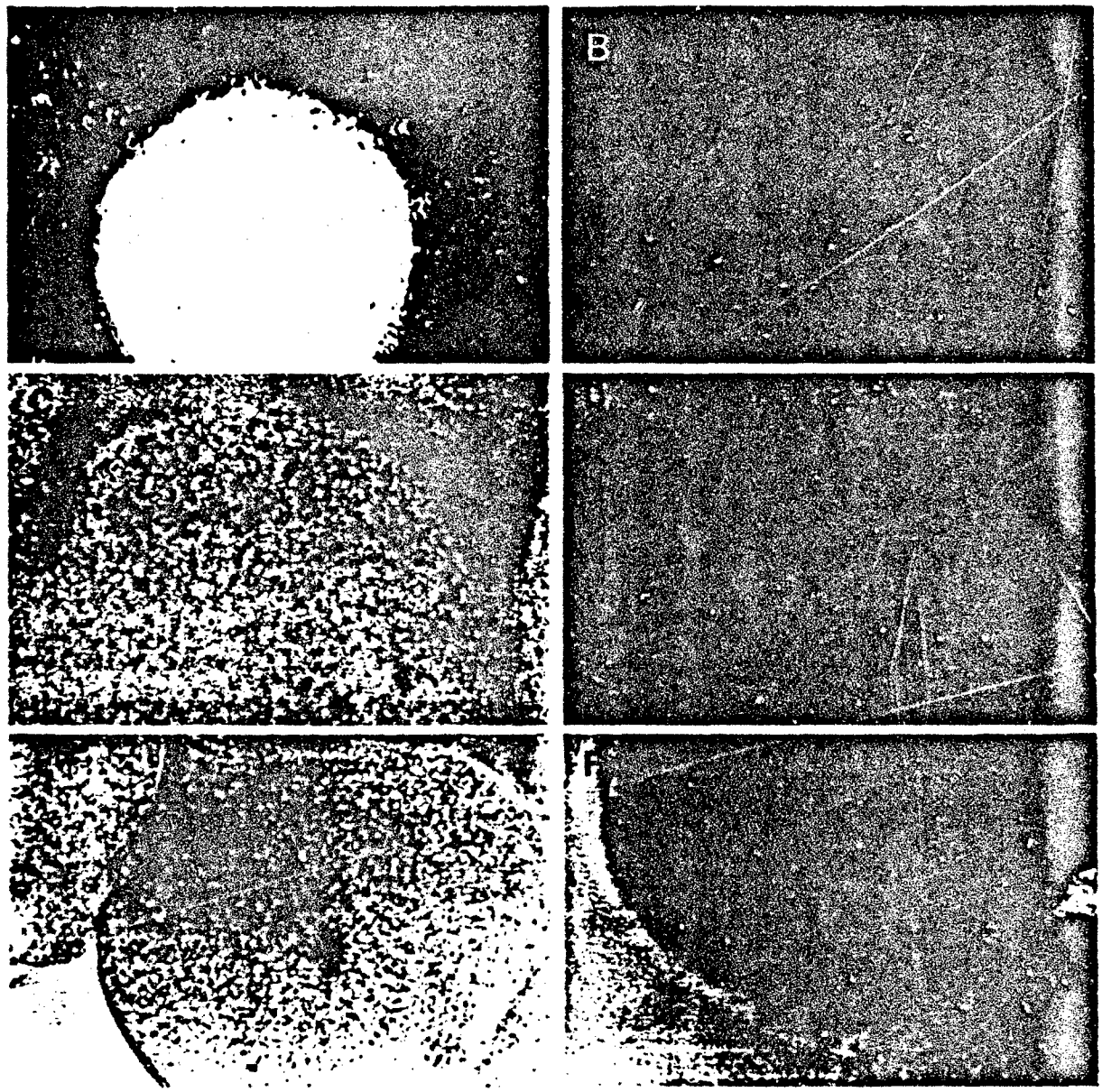
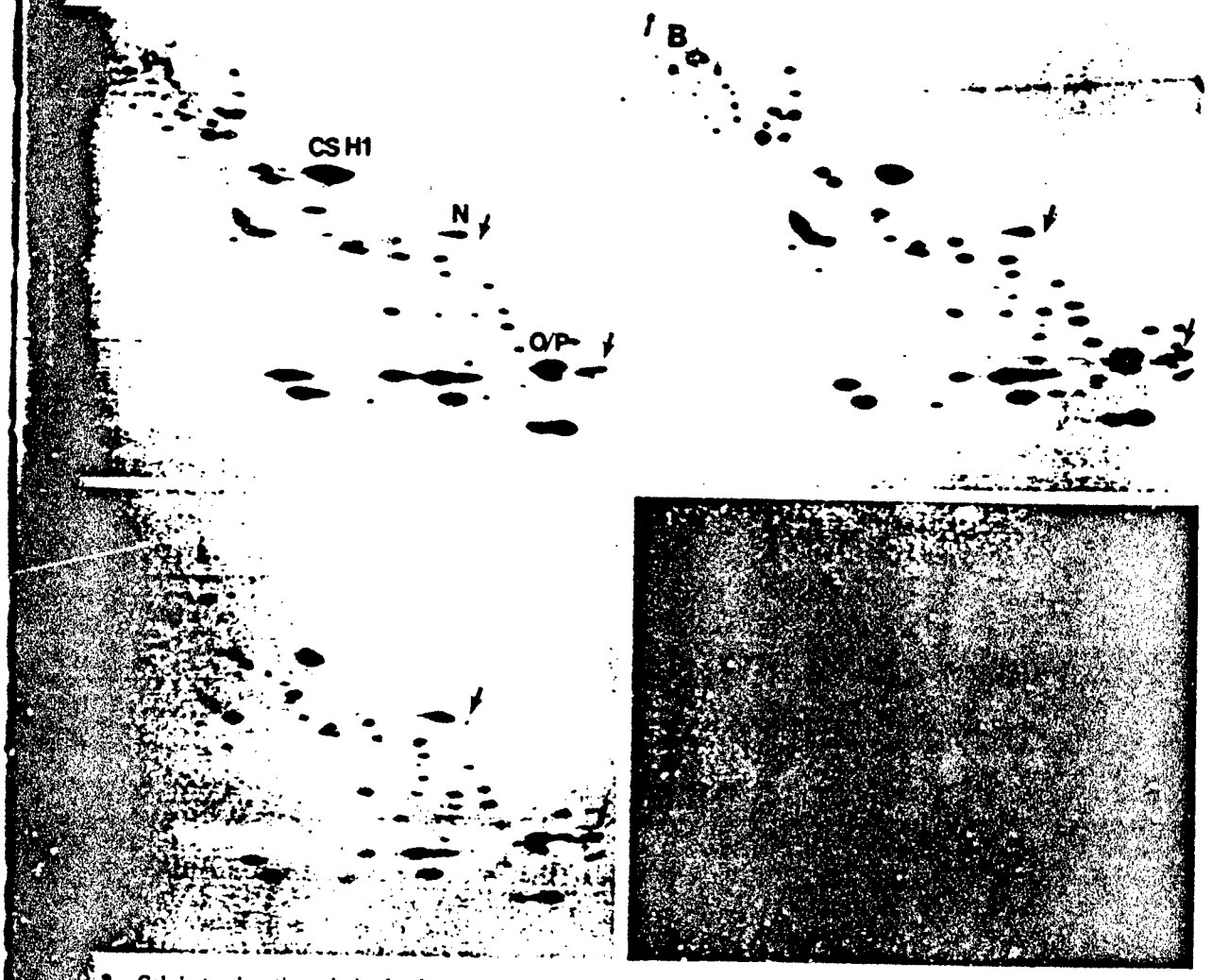


FIG. 2. Effects of 6DMAP on male pronuclear decondensation in polyspermic sea urchin eggs. (A, B) Uptake of sperm. Eggs were prelabeled with Hoechst 33334 prior to 1 m*M* 6DMAP treatment and polyspermic fertilization. Same egg photographed with phase contrast (A), showing large numbers of external sperm, and with fluorescence (B), showing several incorporated but condensed sperm nuclei in the egg at 20 min postfertilization. (C, D) Lack of reversal of inhibition by prior egg activation. Cultures were inhibited with 1 m*M* 6DMAP except one (D) was activated with seawater adjusted to pH 9 with NH_4OH for 20 min prior to fertilization, the last 10 min of which included 6DMAP. Control treated with seawater for 10 min, then N9-6DMAP for 10 min. Both cultures were fixed at 60 min postfertilization. (E, F) Appearance of nuclei just prior to isolation. Nuclei from polyspermic eggs washed at 25 min postfertilization with cold 1 *M* glucose (10 min wash time), just before lysis into isolation buffer. Inhibited nuclei retained their original appearance throughout. Male pronuclei from control polyspermic eggs are somewhat less decondensed than those from monospermic eggs. In E, two male pronuclei are in the same plane of focus as the female large pronucleus. (E) Uninhibited control; (F) inhibited with 1 m*M* 6DMAP.

H2B and contaminants from egg cytoplasm run [3]. Judging from the ratios of O/P to other core histones, it is likely that the spot indicated by the lower arrow in Fig. 3D is largely unmodified Sp H2B, but those in Figs. 3A–3C are largely contaminants. In any event, complete conversion of Sp histones (especially Sp H2Bs) to the



3. Gel electrophoretic analysis of male pronuclear histones of 6DMAP inhibited eggs. First dimension contains acid–urea–Triton X-econd dimension 0.1% SDS. (A) No inhibitor, average degree of polyspermy $\bar{n} = 13 \pm 4$; (B) 600 μM 6DMAP, $\bar{n} = 14 \pm 3$; (C) 1 mM P, $\bar{n} = 14 \pm 5$; (D) 3 mM 6DMAP, $\bar{n} = 13 \pm 5$. Embryos were washed in cold glucose at 25 min and lysed at 35 min postfertilization. s indicate positions where Sp H1 and Sp H2B markers run.

ified forms may be somewhat retarded under very h 6DMAP conditions, but at 1 mM 6DMAP, Sp his- conversion is virtually complete in the conical, con- ed pronuclei.

Since 6DMAP has been reported to be a kinase inhibi- but the modified sperm histones are believed to de- from phosphorylation of Sp histones, a culture was ated with 1 mM 6DMAP as above, and labeled with P]orthophosphate from 20- to 30-min postfertiliza- ion. Pronuclear histones from inhibited and control cul- es are shown in Fig. 4. The cultures were similar in ee of polyspermy (male pronuclei/egg = 21 ± 4 for rol, 25 ± 6 for 6DMAP). The stained gels show sim- histone patterns (Figs. 4A and 4B). Again, conver- o N and O/P are virtually complete. The corre- sponding autoradiograms show that the proteins N and O/P are indeed phosphorylated in the presence (Fig. 4D) or absence (Fig. 4C) of 6DMAP. In fact, compared to the control, Sp histones from the 6DMAP-treated culture exhibit somewhat higher specific activities. Most other proteins on the gel showed lower levels of phosphate incorporation indicating a general inhibition of phosphorylation. Since nuclei were conical in the inhibited culture, we conclude 6DMAP does not suppress decondensation by inhibiting phosphorylation of Sp histones.

DISCUSSION

6DMAP and Sp histone phosphorylation. Loss of the sperm nucleus conical morphology and chromatin de-

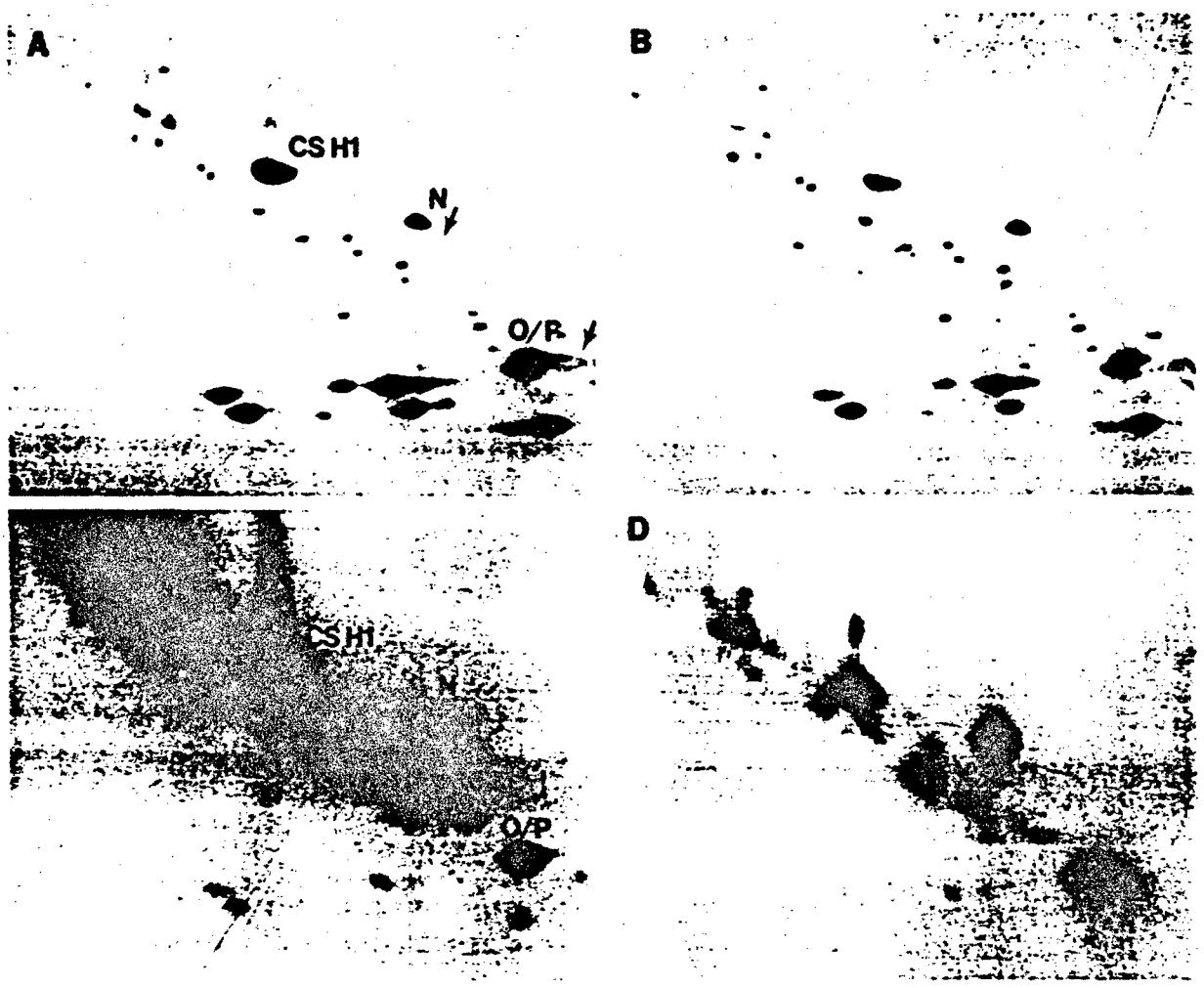


FIG. 4. Phosphorylation of Sp histones in the presence of 6DMAP. Two-dimensional gel electrophoresis as in Fig. 3. (A, C) Untreated control, $\bar{n}$ = 21 ± 4; (B, D) 1 m*M* 6DMAP-treated, $\bar{n}$ = 25 ± 6. [^{32}P]orthophosphate was added at 20 min, embryos were lysed at 35 min postfertilization, and nuclei were isolated. C and D are autoradiograms of gels in A and B, respectively.

condensation precede, and perhaps are required for, genetic reactivation of the sea urchin male pronucleus. DNA and RNA synthesis are initiated with male pronuclear chromatin of histone composition and physical structure very similar to those of genetically active female pronuclei or spermatid nuclei [25, 26]. Genetic reactivation of the male pronuclei follows after the phosphorylation of Sp histone variants and chromatin decondensation [3, 26].

It is clear from our results that the phosphorylation of Sp histones is not sufficient for male pronuclear decondensation. Phosphorylation can take place in a fully condensed pronucleus which has maintained its conical morphology. Indeed, the specific activity of phosphorylated Sp histones under these conditions is somewhat higher than that of controls. Thus 6DMAP, which substantially inhibits kinase activity in eggs (Ref. 11; Fig. 4, this paper) does not inhibit the kinase responsible for Sp histone phosphorylation. This kinase has substrate specificity resembling that of M-phase histone kinase [5, 17].

6DMAP and egg activation. How 6DMAP exerts its effects prior to or at fertilization is not clear. Previous studies using 6DMAP on echinoderm eggs showed that it is a potent reversible inhibitor of meiotic progression and mitotic cycling [11, 14]. Relatively few experiments have explored treatment with 6DMAP prior to fertilization in the sea urchin, whose eggs are fertilized after completion of both meiotic divisions.

Known early triggers at fertilization involve phosphatidylinositol pathways which result in cleavage of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-tri-

phosphate (IP_3) and diacylglycerol. Since IP_3 is believed to trigger Ca^{2+} release, yet fertilization membrane elevation, which is initiated by Ca^{2+} release, occurs normally in the presence of 6DMAP, the IP_3 pathway seems unobstructed.

Since IP_3 and diacylglycerol are produced in the same reaction, we can assume diacylglycerol production is normal. Diacylglycerol is thought to lead to alkalinization of egg cytoplasm at 1–5 min postfertilization, through activation of protein kinase C, which in turn activates a Na^+/H^+ carrier [27, 28]. A block following diacylglycerol formation might lead to inhibition of decondensation. However, we find that treatment with N8 or N9 for 10 min prior to 6DMAP treatment, which should be sufficient for artificial cytoplasmic alkalinization, does not appear to relieve the 6DMAP block. It has been reported that 6DMAP does not alter cAMP levels in sea urchin eggs [13], so cAMP-dependent pathways are also unlikely candidates for the mediation of the 6DMAP effects.

6DMAP and MPF activity. Although the phosphorylation of Sp histones at fertilization might be considered a special case of nuclear protein modification in oocytes, there are several reasons to believe it shares some aspects in common with modifications occurring at mitosis: (1) tetrapeptide phosphorylation target sequences of Sp histones following fertilization and somatic H1's at mitosis are identical [5]; (2) the behavior of prematurely condensed sperm chromosomes and mitotic chromosomes (response to cytoplasmic signals and phosphorylation of H1 histones) is similar [29, 30]; and (3) chromosome condensation/decondensation is controlled by MPF for mitotic and meiotic chromosomes in somatic cells or oocytes [31].

Recent studies suggest that cell cycle progression in early embryos depends on MPF activity which may involve at least two components, a kinase capable of phosphorylating histone H1 [15, 16, 32] and cyclin, a protein constitutively synthesized and periodically degraded [33, 34]. In sea urchin eggs, cyclin is a component of the M-phase histone kinase [35].

H1 kinase activity in the mitotic cycles of early sea urchin embryos was measured by Meijer and Pondaven [24]. This activity resembles the growth-associated kinase activity thought to be responsible in this and other cell types for H1 phosphorylation [36]. Meijer and Pondaven [24] found that H1 kinase was inhibited by 6DMAP by more than 90% in the range 600–1000 μM, the range found to be effective at preventing pronuclear decondensation in our study. Ammonia at pH 9 but not at pH 8 triggered H1 kinase cycling; Ca^{2+} ionophore A23187 could not. Most interestingly, the authors reported a transient peak of kinase activity appearing just after fertilization (Fig. 1 of Ref. [24]). The kinetics make this activity a likely candidate for the Sp histone kinase which we previously suggested to be the same as the M-phase histone kinase [5]. However, we were not able to alter 6DMAP inhibition by pretreatment of eggs with N-9, which should initiate H1 kinase cycling [24] and cyclin synthesis [37].

Neant *et al.* [14] reported that although 6DMAP added at 30 min postfertilization inhibited cyclic protein phosphorylation without affecting cyclin synthesis and degradation, when it was added before fertilization, it affected cycling of cyclin. Although data for the latter effect were not given, it was implied that cyclin was synthesized but not degraded. It is not apparent how inhibition of cyclin degradation which occurs at the first mitosis could impede pronuclear decondensation which occurs within a few minutes of fertilization, unless levels of cyclin are high or build up in 6DMAP-inhibited oocytes before fertilization. The egg might then be arrested and incapable of exiting this state. Since female pronuclear chromatin is decondensed throughout the period when male pronuclei normally swell, a general arrest in some sort of chromosome-condensing cytoplasmic state seems unlikely.

Sp histone phosphorylation and chromatin decondensation. It is commonly suggested that the M-phase kinase controls chromosome condensation through phosphorylation of H1 [36, 38]. In male pronuclei, however, the effect is opposite; phosphorylation is correlated with decondensation. We believe that phosphorylation of histones modulates ionic interaction with DNA which permits other factors [7, 8] to control the state of condensation of the chromatin. Several observations support this view. In the first mitotic cycle of sea urchin embryos, inhibition of chromosome condensation by the protein synthesis inhibitor emetine does not affect phosphorylation of CS H1 [30]. In HTC cells, inhibition of dephosphorylation with $ZnCl_2$ does not inhibit chromosome decondensation [39]. During sea urchin spermiogenesis, virtually all chromatin condensation takes place before histone dephosphorylation of Sp H1 and Sp H2B [2].

The present study indicates that Sp histone phosphorylation is not sufficient for male pronuclear chromatin decondensation following fertilization. It is not clear whether decondensation *in vivo* can occur in the absence of Sp histone phosphorylation, but it has yet to be observed. An effective reversible inhibitor of Sp histone kinase would aid in the analysis of the requirement for phosphorylation.

It seems likely that the function of Sp histone variants is to stabilize the structure of the mature sperm nucleus. In this way, their effects are analogous to the disulfide-linked protamines of mammals [1]. In mammals there is good evidence both *in vivo* and *in vitro* that disulfide reduction permits decondensation [40]. It is possible that phosphorylation serves the same function in sea urchin sperm histones as reduction in mammalian prot-

amines, and that once this event has occurred, alterations of the degree of chromatin swelling can proceed by other cellular mechanisms.

The occurrence of the tetrapeptide ser-pro adjacent to two basic amino acids in viral proteins, unique sperm proteins of invertebrates and vertebrates, and plant histones suggests that reversible phosphorylation may be involved in many different schemes for packaging/unpackaging chromosomes [5]. Its occurrence in a negative regulator of human chromosome condensation lends further support to this interpretation [8].

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Note added in proof. F. C. Luca and J. V. Ruderman (41) have reported that 6DMAP and *N*-ethylmaleimide are members of a small group of inhibitors of cyclin destruction *in vitro*. Since 6DMAP (this study) and NEM (Luttmer and Longo, Ref. [10]) are potent inhibitors of male pronuclear decondensation in sea urchin eggs, we suggest that cyclin levels (and therefore H1 kinase activity) may be high in the unfertilized egg and cyclin destruction at fertilization might be required for decondensation.

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THE PATERNAL CENTROSOME DIRECTS THE POLARITY OF EARLY PATTERN FORMATION IN THE FERTILIZED ASCIDIA CERATODES EGG

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SUMMARY

In this study, we have documented the configuration of microtubules and centrosomes in early development of the phlebobranch Ascidia ceratodes. Centrosomes were detected using a polyclonal antiserum to the centrin molecule (1). We show here that the sperm contributes the active centrosome in development. Following the first phase of ooplasmic segregation, the paternal centrosome was initially maximally compacted and nucleated a tiny sperm aster at the vegetal pole which subsequently enlarged. However, anti-centrin reactivity was absent from the unfertilized egg. Following the completion of meiosis, the centrosome nucleated microtubules that permeated the entire zygote reaching the cortex at the animal pole. Simultaneously, the sperm aster migrated sub-equatorially to the future posterior pole of the embryo, parallel to its cortex. Following centrosome duplication, pronuclear fusion occurred and the mitotic apparatus then migrated to a sub-eccentric position in the cytoplasm. As the mitotic asters enlarged, both poles expanded. At cytokinesis and interphase of the 2-cell stage, the centrosome displayed a perinuclear localization. These findings reveal the existence of a centrosome cycle in ascidians, and suggest that changes in shapes of paternally-derived centrosomes may specify the spatial topography of microtubules they nucleate.

INTRODUCTION

More than eighty years have elapsed since Conklin first published his now illustrious monograph on the cell lineage of the ascidian egg (2). He observed that following fertilization, the eggs of Styela partita underwent a spectacular series of cytoplasmic rearrangements, later to be collectively defined as ooplasmic segregation (3). The ascidian egg is said to be "mosaic", in that the primary lineages (muscle/mesenchyme, notochord, endoderm and ectoderm) are rigorously determined by a phenomenon known as cytoplasmic localization. Conklin first demonstrated this mechanism by isolating or ablating selected blastomeres in early cleavage stages. Whittaker later confirmed it (4) using cleavage-arrested embryos. The appearance of lineage-specific markers in selected blastomere subsets supported the hypothesis that cytoplasmic determinants specifying the expression of these markers became

segregated into particular cell lineages during development. It is believed that these "determinants" may be maternally inherited mRNAs which activate groups of genes in specific lineages (3). A variety of studies have implicated various cytoskeletal elements in the mediation of cytoplasmic segregation, including microfilaments in ascidians (3,5) and nematodes (6), and microtubules in nemertines (7) and amphibians (8). Sawada (5) and Jeffery (3) have proposed a model in which a cortical contraction of microfilaments may provide the motive force during the first phase of ooplasmic segregation, in which the sperm and myoplasmic material accumulate at the vegetal pole. The second phase is characterized by the formation of the various territories of the future tadpole. Here, we have documented the organization and structural changes which take place in microtubules and centrosomes of unfertilized eggs and early embryos.

MATERIALS AND METHODS

ANIMALS

Ascidia ceratodes was collected off the floating docks at Fisherman's Wharf in Monterey Bay, CA. The eggs which lay gravid in the female gonoduct, and which arrested at metaphase I of meiosis, were surgically removed and placed in Sterile 0.2 micron Filtered Sea Water (SFSW) prior to each experiment. Sperm was collected dry, usually from a different animal, and stored at 4°C until needed.

DECHORIONATION PROCEDURE AND FERTILIZATION

Ascidia eggs were dechorionated following a modification of the procedure described by Mita-Miyazawa (9), fertilized with a preactivated sperm suspension, and incubated at 16°C. Normal development ensued and swimming tadpoles complete with chordate morphology were obtained 18-20 hours later.

DETERGENT EXTRACTION AND IMMUNOFLUORESCENCE OF EMBRYOS

Unfertilized eggs and dechorionated embryos of various stages were briefly immersed in two volumes of microtubule-stabilizing buffer (MSB: 0.3M potassium gluconate, 0.33 M glycine, 5mM EGTA, 2mM magnesium sulfate, 10mM sodium chloride, 5% glycerol and 10mM MES, pH=6.0) which had been overlaid in a conical centrifuge tube containing 10 volumes of extraction buffer (MSB + 0.1% triton X-100, pH=6.0). The eggs were extracted for one hour, after which they were placed onto Poly-L-Lysine-coated (390,000 MW, Sigma) chamber slides (Nunc Inc., Naperville) for 20 minutes. The slides were fixed with cold (-20°C) methanol for 15 minutes and then gradually rehydrated with phosphate-buffered saline pH=7.2 (PBS). The eggs were then incubated in a blocking

solution containing 5% normal goat serum/PBS for 30 minutes, after which anti-mouse beta-tubulin monoclonal antibody (Amersham) and centrosome antiserum (kindly provided by Dr. Jeff Salisbury, Case Western Reserve University, Cleveland) were added at appropriate dilutions and incubated for 1 and 16 hours respectively, followed by four washes of 5 minutes each. The antibody detects the centrin molecule in plant and animal cells (1). Secondary antibodies which consisted of Fluoresceinated goat anti-mouse IgG and/or Texas Red-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, PA) were incubated for 1 hour, and washed as previously. In the final step, the DNA was labeled with the fluorescent dye DAPI (1ug/ml) for 2.5 minutes and rinsed twice for 5 minutes. The slides were mounted with a solution containing 90% glycerol, 10% PBS and 100mg/ml DABCO (Sigma), and examined under a Zeiss epifluorescence microscope.

RESULTS

NORMAL DEVELOPMENT OCCURS FOLLOWING CHEMICAL DECHORIONATION

The unfertilized egg of *Ascidia ceratodes* is enclosed within an inner layer of test cells firmly attached to the plasma membrane, and an outer perivitelline space that is itself covered by a thick proteinaceous chorion upon which rests a layer of follicle cells. In order to investigate cytoskeletal organization and rearrangement in the early embryo, these layers had to be removed without impairing development. Eggs were thus chemically dechorionated and various batches were then inseminated with preactivated sperm suspensions. As a result, the denuded eggs initially exhibited a series of rotatory movements, and 5 minutes following insemination a cortical contraction developed along the animal/vegetal axis which lasted two minutes (data not shown). First and second polar bodies occurred at 12 and 22 minutes respectively, and embryos underwent cytokinesis 48-50 minutes following insemination followed by cleavages every 20 minutes thereafter. Embryos reached the tailbud stage around 7 hours of developmental time and completed embryonic development around the same time as chorionated embryos, namely at 18-20 hours. These findings, which will be described in full in a separate manuscript, demonstrate that normal fertilization and development occurred in embryos deprived of their chorion/test cell layers.

ANTI-CENTRIN REACTIVITY IS SELECTIVE TO THE PATERNAL CENTROSOME

The unfertilized egg of Ascidia contains a network of microtubules throughout the cytoplasm, many of which reach the cortex. The fusiform meiotic apparatus lays parallel to the cortex at the animal pole in meiotic

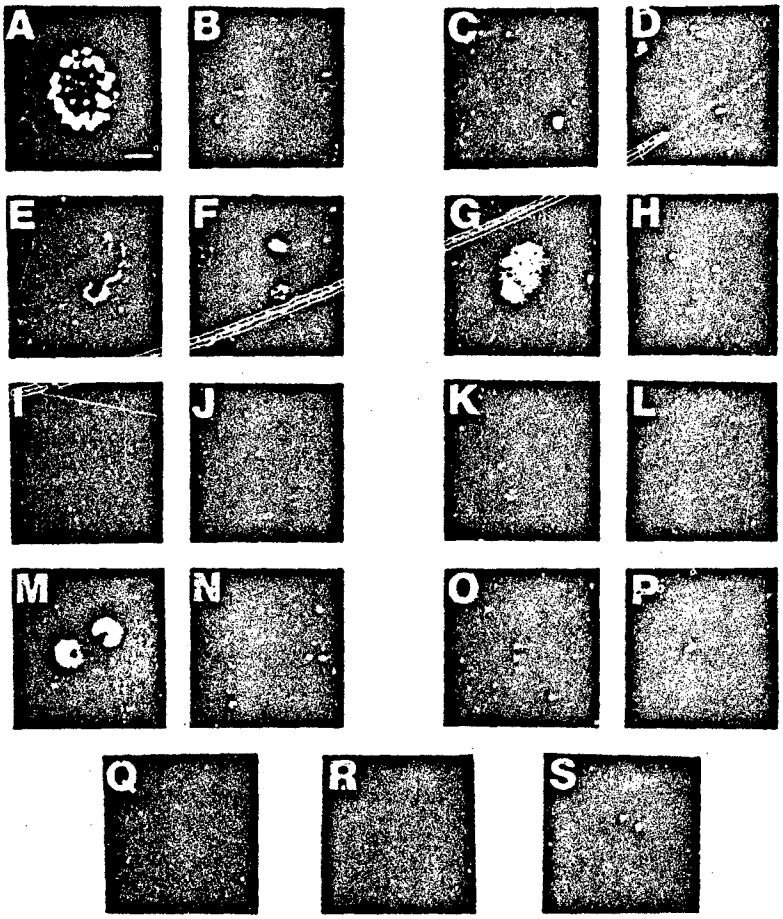

Figure 1. Microtubules and Centrosomes in unfertilized, fertilized and mitotic ascidian eggs. Multiple cytasters are only found in the unfertilized egg (A), while the chromosomes arrest in metaphase I of meiosis (B). Following fertilization, the compact centrosome nucleates a small aster at the vegetal pole (C) with closely apposed male pronucleus (D). Following meiosis, the microtubules are seen radiating throughout the entire zygote nucleated by an arc-shaped centrosome (E,G), which has migrated sub-equatorially to the posterior region. Panel E shows an embryo viewed from the vegetal pole, and female pronuclei can be seen in different focal planes (F,H). The paternal centrosome duplicates to form mitotic poles (I,J), and pronuclear fusion occurs at mitotic prophase (K,L). At metaphase, the mitotic apparatus has migrated to a sub-eccentric position in the zygote (M,N) and poles enlarge during anaphase (O,P). At cytokinesis (Q-S), microtubules radiate to the cortex (Q) and centrosomes have a perinuclear localization (R). Panels (A-D, E-F and I-P) and G-H are double-stained for microtubules and DNA, and centrosomes and DNA respectively, while panels Q-S are triple-stained for centrosomes, microtubules and DNA. The bar represents 36 microns.

metaphase I configuration. In several batches of eggs, multiple cytasters permeated the entire egg cytoplasm (fig.1 A,B). Following fertilization, all cytasters disappeared as eggs underwent the first phase of ooplasmic segregation. At 10 minutes, a minute sperm aster appeared at the vegetal pole with a posteriorly apposed and condensed male pronucleus (fig.1 C,D). Anti-centrin reactivity was restricted to and nucleated the sperm aster (data not shown). Surprisingly, the meiotic apparatus, although bearing functional poles, did not stain with the centrin antiserum nor did the cytasters of the unfertilized egg. These results indicated that maternal and paternal MTOC differed structurally.

By 18 minutes, the sperm aster had enlarged and the male pronucleus had begun to decondense slightly. At 25 minutes, following emission of the second polar body, various events occurred in concert: first, the sperm aster now extended along the entire embryo and microtubules were seen radiating parallel to the cortex (fig.1 E); second, the decondensing female pronucleus initiated its migration towards its male counterpart (Fig.1 F), a process that required intact microtubules (6 and personal communication); third, the configuration of the paternal centrosome was that of an arc which assumed an anterior position relative to the male pronucleus (Fig.1 G,H). Microtubule bundles also co-localized to this centrosomal area (fig.1 E); finally, the sperm aster initiated a migration along the cortex to a sub-equatorial localization which corresponded to the future posterior pole of the embryo. At 33 minutes, the centrosome separated to give rise to the mitotic poles (figl I,J), after which pronuclear fusion occurred at 37 minutes (Fig.1 K,L). When the embryo reached metaphase at 42 minutes, the mitotic apparatus had migrated toward the center of the embryo, (Fig.1 M,N). At anaphase, the poles widened accompanied by an increase in size of astral microtubules (fig.1 O,P). Between 48 and 50 minutes, the zygotes initiated cytokinesis and reached the 2-cell stage at 52 minutes. Microtubules were observed radiating throughout both blastomeres and centrosomes displayed a perinuclear localization (figure 1 Q,R,S).

DISCUSSION

The main conclusions from the present study are that i) ascidians follow the paternal mode of centrosomal inheritance, and that ii) centrosomes can assume different shapes following fertilization and during the mitotic cycle. Following sperm incorporation, the centrosome is initially compact and nucleates a tiny aster at the vegetal pole. As the sperm aster grows, the male pronucleus decondenses and the centrosome expands. Conklin had originally noted (2) that following its entry into the egg cytoplasm, the sperm head rotated such that the centrosome assumed an anterior position

relative to the male pronucleus. The results of this study have confirmed and extended this observation. At 25 minutes, following meiosis, the sperm centrosome assumed an arc configuration and microtubule bundles spanned the entire embryo, as well as co-localizing to the centrosomal area. The sperm aster also initiated a migration along the cortex towards the future posterior pole of the embryo. In addition, co-localization of microtubules and anti-centrin reactive material occured at cytokinesis and interphase of the 2-cell-stage embryo.

Recently, two groups reported (10,11,12) that mesodermal crescent formation was dependent on the migration of the sperm aster during the second phase of ooplasmic segregation, and that microtubule inhibitors prevented this step in Molgula occidentalis embryos (11) . In addition, Bates and Jeffery (13) have reported that axial determinants are transiently localized at the vegetal pole between the first and second phase of ooplasmic segregation in Styela plicata. The findings presented here suggest that changes in shape of the paternal centrosome may govern changes in the configuration and position of the microtubules it nucleates. The reorganization of the microtubule network by the male centrosome suggests that it may be a key participant in the establishment of cytoplasmic territories in early development, as was recently proposed in pole cell formation of Drosophila embryos (14). Raff and Glover found that when embryos were treated with aphidicolin in the early pre-cleavage stages of drosophila emb-yos, DNA synthesis was prevented while centrosomal replication occured normally. Remarkably, centrosomes migrated to the posterior pole and initiated pole cell formation in the absence of nuclei (14). In ascidians, the sperm centrosome could conceivably be involved in specifying the polarity of microtubule tracks onto which cytoplasmic segregation would occur. Whether it contributes some element of specificity (attachement of maternal mRNAs at non-random sites along microtubules), is currently unknown. Direct demonstration of myoplasmic elements using specific antibodies (15) should reveal the extent of segregation of these non-cytoskeletal elements.

In conclusion, we suggest that the paternal centrosome undergoes changes in shapes which govern microtubule topography. The microtubules may in turn serve as tracks onto which cytoplasmic segregation occurs in the early ascidian embryo. Finally, the results demonstrate the existence of a "centrosome cycle" in ascidians, as originally described in the sea urchin (16), and attests to the remarkable conservation of the centrin analogue throughout evolution.

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Marine Biology

Light regulation of nitrate reductase in *Ulva fenestrata* (Chlorophyceae)

I. Influence of light regimes on nitrate reductase activity*

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Abstract. Observations were made on the behavior of nitrate reductase activity in the green alga *Ulva fenestrata* under controlled light:dark regimes. The activity of nitrate reductase (NR) was examined in response to normal seasonal photoperiods as well as in response to shortened or extended periods of darkness. NR activity exhibits a light-dependent diurnal rhythm under both normal summer and winter photoperiods, with a maximum in the early morning (2 to 2.5 h after the start of illumination). This peak of activity is followed by a lower steady-state level of activity which is sustained throughout the light period. There is a sustained minimal level of activity in darkness. The morning peak in activity is always observed as long as tissue is illuminated, irrespective of the previous light or dark treatments. As such, it appears that nitrate reductase activity in *U. fenestrata* is under circadian control. There is no major difference in the NR activity pattern between summer and winter plants, except that the peak activity values in winter plants are consistently much higher (5 times) than in summer plants. The study also suggests that illumination prior to the normal start of photoperiod triggers a different set of regulatory mechanisms, indicating that the physiological state of plants is important in dictating the NR activity response to illumination.

Introduction

Nitrate reductase (NR) catalyzes the reduction of nitrate to nitrite, the first step and a potential regulatory site for nitrate assimilation in plants (Beevers and Hageman 1969). NR activity is influenced by a variety of environmental factors, including photon flux level, light quality, substrate concentration, ammonium, molybdenum, iron, and other regulators of growth (Campbell 1988). In addition, studies have indicated a close association between metabolism of photosynthetic products and nitrate reduction in photosynthetic tissues. The coupling of nitrate and carbon metabolisms arises from the reducing power and carbon-skeleton requirements for the synthesis of amino acids from ammonium produced during nitrate assimilation (Rathnam 1978). Therefore, the role of light in controlling the behavior of NR is not simple and requires the investigation of several different metabolic compartments.

NR behavior under various light regimes has been studied in several higher plant species (e.g. Shivashankar and Rajgopal 1983, Campbell and Smarrelli 1986, Galangau et al. 1988), and in some macrophytic and planktonic algae (e.g. Tischner and Huttermann 1978, Weidner and Kiefer 1981, Davison and Stewart 1984, Tischner 1984, Velasco et al. 1989). These studies have shown that NR activity, enzyme and/or mRNA levels may change in response to the light regime, and that some of these features appear to show circadian or ultraradian rhythms (cf. Edmunds 1988). These data, however, are equivocal as to whether NR activity is directly sensitive to light and, if so, at what level, i.e., post-translational, translational or transcriptional. A post-translational control might entail enzyme activation-inactivation, while translational or transcriptional regulation implies *de novo* synthesis of this protein and or differential expression of NR genes. Marine macrophytes occupy a major ecological domain which is very different from those of higher plants and phytoplankton; therefore, the regulation and activity patterns of NR might deviate significantly from that known for other autotrophs. Unfortunately, at present there is a paucity of information on the regulation of NR activity and NO_3 assimilation in macroalgae, although a few studies have been done on the spatial distributions of NR activity within the thallus, such as Davison and Stewart's study (1984) which showed an in vivo activity variation of 0.05 to 0.3 μmol NO_2 g^{-1} min^{-1} along the thallus of *Laminaria digitata*.

The present investigation sought to characterize the behavior of NR in the marine macrophyte *Ulva fenestrata*. *U. fenestrata* is a benthic green alga possessing a distromatic thallus. It is found in the low intertidal zone

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and is often conspicuous on rocky shores on both the northeast and west coasts of the United States. The low intertidal habitat is characterized by regular, tidal fluxes that may expose plants to desiccation and potential N-limitation during low tides (Thomas and Turpin 1980, Gerard 1982). Previous studies showed that photosynthetic capacity in *Ulva* spp. displays rhythmic patterns that seem to correspond to a circadian tempo (Ramus 1981), which may be controlled at the level of photosynthetic electron transport between plastoquinone and Photosystem I (Mishkind et al. 1979). In addition, there is a strict diel pattern of chloroplast migration between the sides and faces of *Ulva* spp. cells (Britz and Briggs 1976), the significance of which is not known (Nultsch et al. 1981).

Here, we examine the role of light as an inducer of NR activity and consider the role of natural and manipulated daily light and dark periods in controlling enzyme activity. In addition, we investigate the role of natural summer and winter photoperiods in regulating the daily pattern of NR activity, since *Ulva fenestrata* in Monterey Bay grow under a temperate regime which is characterized by distinctive seasonality in photoperiod.

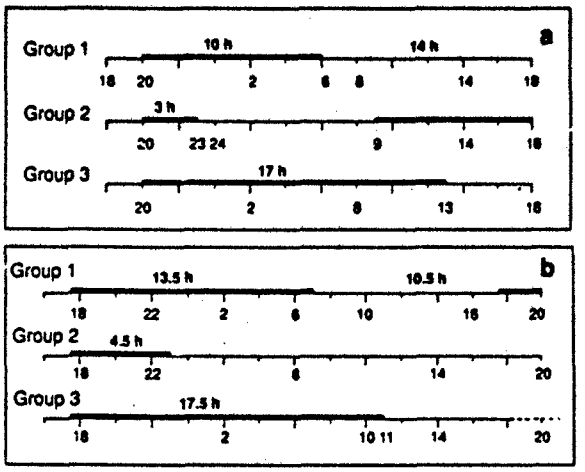

Fig. 1. Photoperiods used to monitor light dependence of nitrate reductase (NR) activity in *Ulva fenestrata*. Light intensity was constant for all light periods. Group 1 represents natural photoperiod for each season, Groups 2 and 3 shortened and extended dark periods, respectively. Values under the respective abscissas show time of day (hrs). Thick bars indicate dark periods. (a) Summer experiment; (b) winter experiment

Materials and methods

Plant material

Ulva fenestrata was collected from an outdoor tank at the Monterey Bay Aquarium, Monterey, California, during summer 1989 and winter 1990. Plants were maintained in enriched seawater with an initial nitrate concentration of 880 μM, at 16 °C ± 2C°, under a photon flux of 200 µmol quanta $m^{-2} s^{-1}$ PAR (photosynthetically active radiation) which saturates growth and photosynthesis in *Ulva* spp. (Arnold and Murray 1980). Light regimes for the summer experiments were 14 h light : 10 h dark and 10.5 h light : 13.5 h dark for the winter experiments. These photoperiods correspond to the natural day lengths during the experimental seasons in Central California. This irradiance level was kept constant during both the maintenance periods (1 d) and experimental periods. After the maintenance period, the plants were divided into three groups corresponding to different light treatments (Fig. 1) – Group 1: control, subjected to natural seasonal photoperiodic duration; Group 2: subjected to a light-interrupted dark period which yielded a dark period of 3 h for summer plants and 4.5 h for winter plants; Group 3: exposed to extended dark periods of 17.0 or 17.5 h.

NR activity assay

NR activity was measured approximately every 2 h during the experiments (see Fig. 1). Sampling intervals were shortened to 0.5 h during the dark-to-light transitions. Tissue samples were taken with a cork borer (1.0 cm diam) 1 h prior to the measurement of enzyme activity. The tissue disks were held in the original culture medium until transfer to the incubation medium. NR activity was measured in vivo by the colorimetric assay technique modified from Brunetti and Hageman (1976) and Dipierro et al. (1977), as described below:

The incubation medium consisted of artificial seawater with 20 mM nitrate, and contained 3.0% (v/v) 1-propanol to increase the permeability of the cell membranes. The concentration of 1-propanol was optimized for the *Ulva fenestrata* tissue. For each reaction, 0.05 g of fresh tissue (three discs) was placed in 5 ml incubation medium and, for each time point, three replicates were used. The incubation proceeded for 1 h in darkness (to prevent the reduction of NO_2^- to NH_4^+), at 16 °C on a shaking table, and subsamples of 1 ml medium were taken at 30 min intervals. Nitrite production was determined colorimetrically using 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-naphtyl-ethylenediamine (NED). Color development was monitored by absorbance at 540 nm with a Hewlett-Packard diode array spectrophotometer (Model 8452A). NR activity was determined by the rate of product formation and corrected for changes in the volume of incubation medium during the sampling period. Values are presented as µmol NO_2 g^{-1} h^{-1}, which equals one unit (U) of NR activity.

Results

NR activity under summer and winter photoperiods

Fig. 2a shows changes in NR activity of *Ulva fenestrata* under summer photoperiods with illumination from 06.00 to 20.00 hrs. Activity increased immediately after illumination, reaching a maximum (0.353 µmol NO_2 g^{-1} h^{-1}) after 2 h, and declined to a plateau at ~0.185 µmol NO_2 g^{-1} h^{-1} for the remainder of the light period (Table 1). The induction rate for the early morning activity maximum was 0.174 U h^{-1}. When illumination ceased, the activity rapidly dropped to a minimum of ~0.006 U (~2% of the maximum activity) within 2 h, and remained at this level until the next illumination period (see Table 1).

The NR activity pattern in plants under natural winter photoperiods is illustrated in Fig. 2b, with illumination occurring between 07.00 and 17.30 hrs. Similar to the summer pattern, an early morning peak in activity was reached ~2.5 h after the artificial sunrise, followed by a decline in rate to a plateau. NR activity was extremely low (~2% of the maximum activity) during the dark period, and there was only one peak of activity in each light : dark cycle.

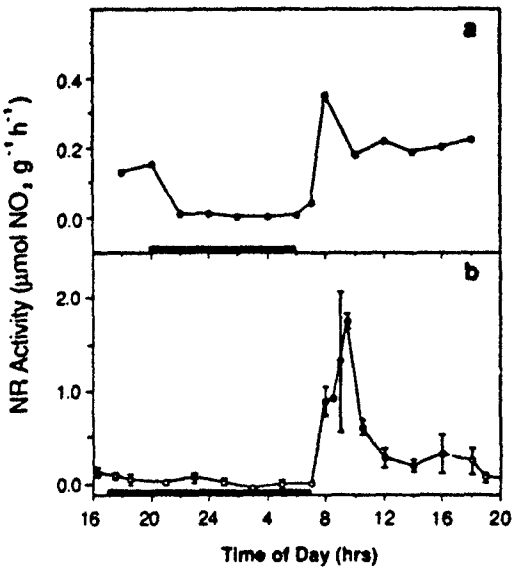

Fig. 2. *Ulva fenestrata.* Diurnal rhythm of in vivo NR activity in plants under natural photoperiods (Group 1 plants); thick bars indicate dark periods. (a) Summer experiment with 14 h light : 10 h dark cycle; induction rate for early morning peak = 0.174 U h^{-1}. (b) Winter experiment with 10.5 h light : 13.5 h dark cycle; induction rate for early morning peak = 0.662 U h^{-1}. Vertical bars = ± SD; $n = 3$

Table 1. *Ulva fenestrata.* Peak, steady-state and dark values of nitrate reductase (NR) activity. Steady-state averages are means (± SD) of plateau values for each photoperiod; dark averages are means (± SD) of values for each dark period

Light treatments	NR activity (μmol NO_2 g^{-1} h^{-1} = U)		
	Maximum	Steady-state average	Dark average
Summer			
normal photoperiods	0.353[a]	0.185 (±0.035) ($n = 7$)	0.006 (±0.04) ($n = 5$)
shortened darkness	0.428[b], 0.511[a]	0.197 (±0.024) ($n = 5$)	0.009 (±0.000) ($n = 2$)
extended darkness	0.412[c]	0.13[c] (±0.045) ($n = 2$)	0.014 (±0.015) ($n = 7$)
Winter			
normal photoperiods	1.759[a]	0.345 (±0.161) ($n = 2$)	0.030 (±0.037) ($n = 7$)
shortened darkness	1.561[b], 1.888[a]	0.921 (±0.385) ($n = 7$)	0.060 (±0.061) ($n = 3$)
extended darkness	0.612[c], 1.45[a]	0.260 (±0.136) ($n = 5$)	−0.008 (±0.044) ($n = 12$)

[a] Early morning peak activity
[b] Light-indiced night peak activity
[c] Peak activity after extended darkness

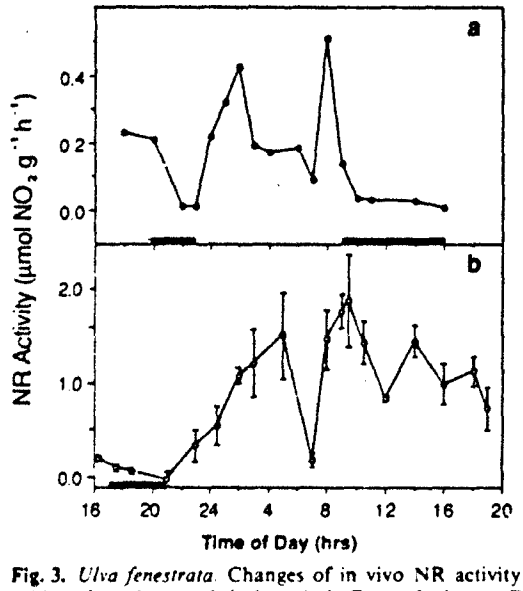

Fig. 3. *Ulva fenestrata.* Changes of in vivo NR activity in plants subjected to shortened dark period (Group 2 plants). First maximum is light-induced peak at night; second maximum is normal early morning peak; thick bars indicate dark periods. (a) Summer experiment; dark period shortened to 3 h, and induction rate of 0.136 U h^{-1} for first peak and 0.424 U h^{-1} for second peak. (b) Winter experiment; dark period shortened to 4.5 h, and induction rate of 0.206 U h^{-1} for first peak and 0.656 U h^{-1} for second peak. Vertical bars = ± SD; $n = 3$

Table 2. *Ulva fenestrata.* Induction rates of in vivo nitrate reductase activity (μmol NO_2 g^{-1} h^{-2} = U h^{-1}) with light-interrupted night period under summer and winter photoperiods (14 h light : 10 h dark and 10.5 h light : 13.5 h dark, respectively); Group 2 plants. The dark duration was 3 h for summer plants, and 4.5 h for winter plants during experiments

Season	NR induction rate		Ratio morning : night
	night max.	morning max.	
Summer, 1989	0.136	0.424	3.1
Winter, 1990	0.206	0.656	3.2

NR activity during light-interrupted night periods

The behavior of NR activity in plants subjected to a light-interrupted night period under summer photoperiods is illustrated in Fig. 3a. Two peaks of activity were observed during the light period. The first occurred at 02.00 hrs, after 3 h of illumination, with an NR activity induction rate of 0.136 U h^{-1} (Table 2); the second maximum occurred at 08.00 hrs, which corresponded to the typical morning peak observed in plants under natural photoperiods characteristic of the same season (see Fig. 2a). This second NR activity maximum had an in-

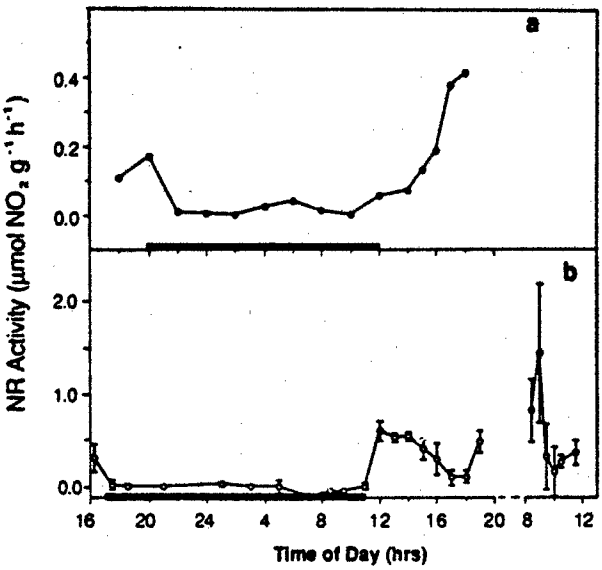

Fig. 4. *Ulva fenestrata.* Changes of in vivo NR activity in plants subjected to extended dark period (Group 3 plants); thick bars indicate dark periods. (a) Summer experiment; induction rate of NR activity upon illumination = 0.078 U h^{-1}. (b) Winter experiment; induction rate of NR activity upon illumination (first peak) = 0.589 U h^{-1}. Samples taken the following morning indicate a recovery of the normal early morning peak with continuous illumination. Vertical bars = ±SD; $n=3$

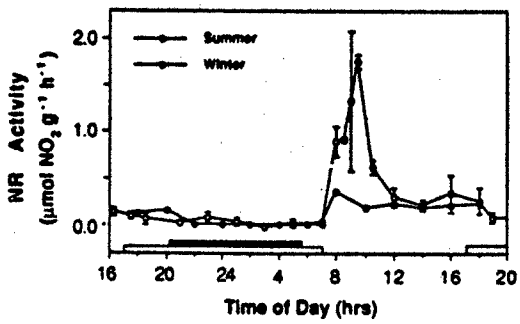

Fig. 5. *Ulva fenestrata.* Comparison of diurnal rhythms of in vivo NR activity between plants under summer and winter photoperiods with light periods of 06.00 to 20.00 hrs and 07.00 to 17.30 hrs, respectively. Thick bars indicate dark periods (filled bar: summer; open bar: winter)

duction rate of 0.424 U h^{-1}, i.e., ~3 times greater than that of the first induction event (Table 2). As in plants under normal summer photoperiods, there was always a significant level of NR activity during the light period and very low activity (< 5% of the steady-state level under light) during the dark period (see Table 1).

The plants subjected to a light-interrupted night photoperiod under winter photoperiods exhibited a behavior similar to that of the summer plants (Fig. 3b). Two peaks of NR activity in one photoperiod were observed – one at 05.00 hrs, 7 h after the onset of illumination, with an induction rate of 0.206 U h^{-1}, the other at the same time as the morning maximum (09.30 hrs) in the control group for the same season, with an induction rate of 0.656 U h^{-1} (Table 2). There was no difference in the timing of the early morning activity peak between plants under different treatments within the same season. Interestingly, the ratio of the induction rates for the premature and normal activity peaks were similar in plants maintained under summer and winter photoperiods (Table 2).

NR activity in extended darkness

Fig. 4a summarizes NR activity in response to extended darkness in summer plants. Activity was minimal (0.014 ± 0.015 μmol NO_2 g^{-1} h^{-1}) until illumination at 13.00 hrs, 7 h after the natural start of the photoperiod, and then increased at a rate of 0.078 U h^{-1}. Fig. 4b illustrates NR activity under a similar treatment for the winter plants. As in the summer plants, the normal early morning peak in activity failed to develop in the absence of illumination. However, when light was given the following morning, NR activity was induced with a maximum achieved at 08.30 hrs. The first peak at 14.00 hrs after extended darkness (4 h longer than the natural dark duration for control plants), was small, and decayed during the subsequent illumination period until ~08.30 hrs the following morning. This pattern was distinctly different from that observed under summer photoperiods (see Fig. 4a).

Despite the variation in lengths of the summer and winter photoperiods, the NR activity patterns exhibited marked constancy in plants maintained under these conditions (Fig. 5). Winter plants exhibited consistently higher NR activity during the light periods than did summer plants.

Discussion

The diurnal pattern of in vivo NR activity in *Ulva fenestrata* under natural photoperiods differs remarkably from the sinusoidal pattern commonly reported for higher plants (Duke et al. 1978, Shivashankar and Rajgopal 1983, Duke and Duke 1984, Lillo and Henriksen 1984, Galangau et al. 1988) and some macrophytic and unicellular algae (Weidner and Kiefer 1981, Davison and Stewart 1984, Velasco et al. 1989, Smith et al. 1992). Generally, the induction of NR activity upon the onset of illumination is much more rapid in *U. fenestrata* (usually within 2 to 2.5 h after illumination) than that reported for other plants (Table 3). In addition, nitrate reduction in *U. fenestrata* does not decline monotonously to minimal levels after the morning activity peak is reached. Instead, the NR activity is sustained at a high steady-state level during the illumination periods. This is the first report of sustained steady-state level of NR activity during light periods in any plant species. Also, instead of showing significant changes in activity during darkness, as observed in other plants, NR activity in *U. fenestrata* remains at a minimal to undetectable level throughout dark periods. The diurnal pattern of in vivo NR activity in *U. fenestrata* does, however, share the single diurnal peak in

Table 3. *Ulva fenestrata*. Timing of diurnal maximum of nitrate reductase activity in different species, i.e., time needed to reach maximal NR activity after start of photoperiod. The light : dark cycles under which these experiments were conducted were all 24 h, except for *Chlorella sorokiniana* which was 12 h (7 h light : 5 h dark)

Species	Time to max. activity (h)	Source
Cocos nuciferas (coconut)	8	Shivashankar and Rajgopal (1983)
Hordeum sp. (barley) *Avena* sp. (oat) *Triticum* sp. (wheat)	~8	Lillo and Henriksen (1984)
Lycopersicon sp. (tomato)	4–5	Galangau et al. (1988)
Chlorella sorokiniana	2	Velarco et al (1989)
Skeletonema costatum	6–8	Smith et al. (1992)
Laminaria digitata	~12 (end of day)	Davison and Stewart (1984)
Ulva fenestrata	2–2.5	Present study

activity observed in previous studies (Table 3). This peak of NR activity is hereafter referred to as the normal daily morning peak.

In plants subjected to a light-interrupted dark period, two peaks in activity in a single photoperiod were observed, whether plants were grown under summer or winter photoperiods (see Fig. 3). The occurrence of the second peak at the normal daily maximum and its markedly greater induction rate compared to the first maximum (the light-induced peak during the night) implicate the existence of circadian or ultraradian processes underlying a diel rhythm in the regulation of nitrate assimilation. Under conditions of an extended dark period, two features are notable. First, the normal morning peak of NR activity is absent when illumination is not supplied during the developing period of this peak, indicating that light is essential for the expression of NR activity. Second, the induction rate of NR activity upon illumination under summer photoperiods is reduced by ~50% compared to that of the early morning peak in plants under normal photoperiods (cf. Fig. 2a and 4a). Most likely, more than simple activation of existing enzymes is involved in the recovery of NR activity, which appears to be suppressed by prolonged darkness.

Although the natural summer and winter photoperiods are quite different from each other, varying from 14 to 10.5 h for the light period and 10 to 13.5 h for the dark period, this difference does not influence the diurnal pattern of NR activity (see Fig. 5). The greater NR activity maximum under winter photoperiods (about 5 times greater than under summer photoperiods) probably reflects the nutritional history of the plants. Nitrate supply is more limited during winter months in the Monterey Bay (unpublished data from the Monterey Bay Aquarium). This observation is consistent with earlier studies indicating that nitrogen limitation can dramatically enhance the capacity for nitrate assimilation (Gerard 1982).

The difference between the summer and winter photoperiods does not seem to influence the patterns of NR activity in plants subjected to light-interrupted dark periods, except that the peak values of NR activity are consistently higher in winter plants than in summer plants. Although the light-induced peak of NR activity at night under winter photoperiods is notably delayed compared to that under summer photoperiods (occurring 7 h instead of 3 h after the onset of the light interruption of the dark period) and the induction rates for peak activities are dramatically higher in winter plants than in summer plants, the ratios between the induction rates of the two NR activity maxima under each seasonal photoperiod are similar (see Table 2). This suggests that the induction of NR activity in response to illumination prior to the normal start of the photoperiod may involve the same processes irrespective of seasonal photoperiods, and that the longer time needed for winter plants to reach the first maximum in activity may simply be due to a higher intrinsic capacity for nitrate reduction in these plants. Failure to develop a normal morning peak by plants treated with extended darkness demonstrates that light is essential for the full expression of NR activity in both seasons.

The present investigation of NR activity in a single temperate, benthic marine macrophyte has yielded an enzymatic activity pattern quite different from the sinusoidal pattern widely described in higher plants, unicellular and macrophytic algae (Table 3). In *Ulva fenestrata*, a diurnal pattern exists in NR activity, which is strictly light-dependent and characterized by an early morning peak followed by a lowered steady-state activity level. This diel pattern is likely driven by some endogenous rhythm, which allows the assimilation of nitrate to reach its climax about 2 h after sunrise irrespective of seasonal photoperiods. In addition, the lag in expression of enzyme activity in response to illumination prior to the normal start of the photoperiod suggests that the abundance of the NR enzyme may also reflect diurnal regulation at the levels of translation and/or transcription in *U. fenestrata*.

Nitrate reductase, as a molybdo-flavoprotein containing a *b*-type cytochrome, is well suited for photoreception. Indeed, several studies have suggested a direct blue light activation of the enzyme activity in green algae which is probably mediated through the flavin moiety on the NR protein (Aparicio et al. 1976, López-Figueroa and Rüdiger 1991). NR has also been implicated as a photoreceptor for some blue light-stimulated responses in *Neurospora crassa*. However, this activity was not associated with light-dependent phase shifting of circadian rhythms in this organism (see Edmunds 1988), indicating that photoreception by NR does not have a direct role in setting the circadian clock.

The precise ecological significance of a circadian control of NR activity in *Ulva fenestrata* is not clear. It seems that the rapid induction of NR activity superimposed on a circadian regulation of nitrate assimilation is of benefit to the plants. Unlike some kelps that can store carbohydrates during periods of nitrogen shortage and use them for nitrate assimilation when nitrate is available (Lobban et al. 1985), the relatively simple cellular organization of

the thallus in *Ulva* spp. probably results in a limited storage capacity for carbohydrates. Thus it may be advantageous for this species to be capable of rapidly utilizing nitrate when light is available. However, the rapid response to and positive correlation of enzymatic activity with light suggests a more stringent light-dependent control of NR in *U. fenestrata*, which could limit its ecological success, especially in areas with seasonal periods of early morning cloud cover. In order to gain more insight into the regulation of nitrate assimilation in algae and to fully appreciate the underlying ecological significance and molecular mechanisms, further studies, especially at the protein and mRNA levels, are needed. Comparative studies of NR behavior in different species and between plants under different physiological states and environmental conditions would also be valuable.

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